

PATENT
Docket No. 511582002500

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Pia M. CHALLITA-EID et al.

Application No.: 10/024,652

Filed: December 17, 2001

For: NUCLEIC ACID AND ENCODED
ZINC TRANSPORTER PROTEIN
ENTITLED 108P5H8 USEFUL IN
TREATMENT AND DETECTION OF
CANCER

Examiner: Bridget E. Bunner

Group Art Unit: 1647

**DECLARATION OF STEVEN B. KANNER
CONCERNING NORMAL TISSUE EXPRESSION**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Steven B. Kanner, declare as follows:

1. I am Director of Cancer Research at Agensys, Inc. In this position I direct research and development to identify and validate novel cancer targets. In addition, I oversee research and development of new cancer therapeutics and diagnostics involving these targets. We investigate the effects of specific genes and gene products on tumor development, growth, and progression. I regularly attend national and international conferences addressing issues in cancer research, conferences where established as well as cutting edge ideas are presented.

I have a Ph.D. in Immunology and Microbiology from the University of Miami, Miami, Florida. In addition, I completed a postdoctoral fellowship in the Cancer Center at the University of Virginia. I have worked in the field of molecular biology at the doctoral level for

51158-2002500

BEST AVAILABLE COPY

over 19 years. Accordingly, I have extensive experience in novel target identification and validation, assay development, monoclonal antibody and small molecule drug discovery, with particular expertise in oncology, immunology and inflammation. A copy of my *curriculum vitae* is attached as Exhibit A.

2. This submission is made in support of the proposition that targeted antitumor therapies are useful even when the targeted protein is expressed on normal tissues, including normal vital organ tissues. A vital organ is one that is necessary to sustain life, such as the heart or liver. A non-vital organ is one that can be removed whereupon the individual is still able to survive. Examples of non-vital organs are ovary, breast, and prostate. Two exemplary proteins will be discussed, HER2/neu and epidermal growth factor receptor (EGFR).

3. Herceptin® is an FDA approved biologic that has as its active ingredient an antibody which is immunoreactive with the protein known synonymously as HER2, HER2/neu, or erb-B2. It is marketed by Genentech and has been a commercially successful antitumor agent. Herceptin net sales reached \$483 million in 2004 and in 2003 net sales were approximately \$425, an increase of 10 percent over sales in 2002 (see Exhibit B). The success of Herceptin is indicated by both the volume and trend of its sales figures. During the period it has been marketed, its sales numbers have consistently increased.

4. Herceptin is FDA approved as a treatment for HER2 positive metastatic breast cancer (see Exhibit C). However, the expression of HER2 is not limited to such tumors. The same protein is expressed in a number of normal tissues.

5. The diverse expression of the HER2/neu protein is indicated in Exhibit D.¹

Exhibit D provides data panels for three different GenBank deposits of HER2/neu sequence. The comparative data in Exhibit D show levels of HER2 above the median in numerous normal tissues. In particular, attention is called to the levels present in kidney and heart. The levels in kidney are consistently shown as higher than those in heart. It is emphasized that these values are for normal tissue; thus these tissues are present in all human recipients of Herceptin.

6. The expression of HER2/neu in a diverse array of normal and malignant tissues was corroborated by studies conducted at Agensys, assignee of the present case, by use of RT-PCR (Exhibit F). Again, significant expression of HER2/neu was noted in heart and kidney. Further corroboration of normal expression of HER2/neu by immunohistochemistry is set forth in Exhibit G. In Exhibit G, positive staining for the presence of HER2/neu was found in normal kidney and colon tissue. Additional confirmation of the presence of HER2/neu in normal kidney is provided by Latif, et al.² As shown in this article (which evaluated whether renal cell carcinoma should be a preferred indication for anti-HER2 antibodies such as Herceptin), both protein and mRNA are produced in benign renal tissues. Notably, HER2/neu protein was strongly overexpressed in benign renal tissue (see page 8, left hand column last paragraph of Latif, et al.).

7. Despite the fact that HER2/neu is expressed in such vital tissues as heart and kidney, Herceptin is FDA-approved, commercially successful, and is very useful as an anti-tumor agent. The effect of Herceptin on cardiac tissue, i.e., "cardiotoxicity," has been a limited

¹ The expression data in Exhibit D was established using gene chip technology; see Su, et al, *PNAS Proc. Natl. Acad. Sci. USA*, Vol. 99, Issue 7, 4465-4470, April 2, 2002, Exhibit E. The results for HER2 are available on the GNF Gene Expression Atlas website <http://expression.gnf.org/cgi-bin/index.cgi>.

² Latif, Z., et al., *B.J.U. International* (2002) 89:5-9 (Exhibit H)

side effect to treatment. When patients were treated with Herceptin alone, significant cardiotoxicity occurred in a very low percentage of patients.

8. Of particular note, although the data shows that kidney tissue exhibits even higher expression than cardiac tissue, nephrotoxicity has not been an appreciable side effect of Herceptin treatment at all. In fact, of the diverse normal tissues in which HER2 is expressed, there is very little occurrence of any side effect. Only cardiac tissue has manifested any appreciable side effect at all. A tissue such as kidney, where HER2/neu expression is appreciable, has not been the locus for any side effect.³

9. Taken together, this documentation establishes that production of a target protein such as HER2/neu on normal tissue, even vital normal tissue, does not defeat the utility of the protein as a therapeutic for certain tumors in which the protein is also expressed.⁴ Such physiologic outcomes, where there is normal tissue expression of a cancer-associated protein target, are not unique to HER2/neu as will be discussed below.

10. Several anti-cancer therapeutic products that target the epidermal growth factor receptor (EGFR) are presently in clinical evaluation.⁵ The rationale for EGFR-targeted anti-cancer treatments is both appreciated and well-accepted.⁶ One such treatment composition is Erbitux™ (also known as cetuximab or C225). The active ingredient in Erbitux is an antibody which is immunoreactive with the EGFR. Erbitux antibody has been shown to block the

³ See Exhibit C, the product information for Herceptin® for an overview of side effect issues

⁴ This is particularly true where the target protein, as with HER2/neu is expressed at higher levels in tumor cells relative to normal tissue. In one embodiment, overexpression in tumor tissue can provide for altered, e.g., enhanced, protein availability for antibody binding.

⁵ Baselga, J., *Oncologist* 7 (supp. 4):2-8 (2002) (Exhibit I)

⁶ Id.

proliferation of various cancer cells.⁷ The successful use of Erbitux is shown, for example, by the net sales in 2004 reaching \$260.8 million. (Exhibit K).

11. EGFR is therefore being used and evaluated as a target for treatment of patients with breast, head and neck, lung, kidney and prostate cancer.⁸ However, the expression of EGFR is not limited to such tumors. This protein is expressed in a diverse array of normal tissues.

12. EGFR protein is extensively expressed in adult humans. It is present on all epithelial and stromal cells, select glial and smooth muscle cells,⁹ oral and laryngeal mucosa¹⁰; brain;¹¹ liver,¹² prostate;¹³ placenta;¹⁴ stomach and colon;¹⁵ and skin.¹⁶ It is to be noted that since it is expressed in these normal tissues, EGFR is present in all human recipients of any EGFR-targeted therapy.

13. Despite the fact that EGFR is expressed in numerous normal tissues, including vital tissues such brain and colon, therapeutics that target EGFR are very useful and are in active development. The only significant side effect of such therapeutics has been on skin as an acneiform rash.¹⁷ This has merely been a side effect to treatment. This side effect is so minor that it is used as an indication that therapeutically effective dosage levels have been achieved.¹⁸ Notably, the side effect is a relatively innocuous signal that an individual has achieved a therapeutically effective dose.

⁷ Busam, et al. Br. J. Derm. 144:1169-1176 at 1169 (2001) (Exhibit J)

⁸ Busam, et al, *supra*

⁹ Wells, A., Int. Biochem. & Cell Biol. 31:637-643 at 640 (1999) (Exhibit L)

¹⁰ Christensen, M., Dan Med Bull. 45(2):121-134 (Apr. 1998) (Exhibit M)

¹¹ Ferrer, et al., Prog. Neurobiol. 49(2):99-123 (Jun 1996) (Exhibit N)

¹² Luwar, et al., Cancer Res. 61:5355-5361 (July 15, 2001) (Exhibit O)

¹³ De Miguel, et al., Cytokine 11(9):722-727 (Sep 1999) (Exhibit P)

¹⁴ Mauro, et al., Repro. Fertil. Devel. 7(6): 1465-1470 (1995) (Exhibit Q)

¹⁵ Challier and Menard, Frontiers in Biosci. 4:87-101 at sections 4.2 and 4.3 (January 15, 1999) (Exhibit R)

¹⁶ Jost, et al. Eur. J. Dermatol. 10(7):505-510 (Oct-Nov 2000); Luwar, et al., Cancer Res. 61:5355-5361 (July 15, 2001) (Exhibit S)

¹⁷ Busam, et al, *supra*; Van Doorn, et al., Br. J. Derm. 147:598-601 (2002) (Exhibit T)

¹⁸ Abgenix Press release 20 Aug 2002 (Exhibit U)

14. The data on EGFR establishes that expression of a target protein such as EGFR in normal tissue, even vital normal tissues, does not preclude the utility of the protein as a therapeutic target for certain tumors in which the protein is also highly expressed.¹⁹

15. In summary, targeted antitumor therapies are useful even when the targeted protein is expressed on normal tissues, even normal vital organ tissues. The ability to use a cancer-associated protein in this manner is not unique to any particular protein. The existence of expression of a protein on a normal tissue, including vital organ tissues, still allows for meaningful and successful use of that protein as a therapeutic target.

¹⁹ See footnote 4, *supra*

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at Santa Monica, California, this 15 day of September 2005.


Steven B. Kanner Ph.D.

STEVEN BRIAN KANNER, PH.D.

Agensys, Inc.
1545 17th Street
Santa Monica, CA 90404
Phone: 310.820.8029 (ext. 104)
Fax: 310.820.8489
E-mail: skanner@agensys.com

PROFILE

Pharmaceutical/Biotechnology research leader with extensive experience in novel target identification and validation, screen development and both small molecule drug and antibody discovery, with expertise in oncology, immunology and inflammation. Self-motivated strategic planner, skilled in motivating, developing, hiring, managing and building scientific teams to expedite novel drug/therapeutic candidate discovery for clinical trial consideration.

PROFESSIONAL EXPERIENCE

AGENSYS, INC.
Santa Monica, CA

2003 -

Director, Cancer Research

Direct a research group including 20 scientists (Ph.D. and research associates) to identify, validate and develop novel targets for the generation of new therapeutics for cancer. Prioritize in-house portfolio for evaluation of targets for either monoclonal antibody development or for alliances for small molecule development or vaccine generation. Report to the Chief Scientific Officer.

- Establish teams for the validation of targets using molecular, biochemical and cellular technologies to evaluate novel genes for identifying new monoclonal antibody based cancer therapies
- Lead joint oversight committees with outside collaborators on alliances for proprietary targets to develop monoclonal antibody, small molecule and vaccine approaches

BRISTOL-MYERS SQUIBB PHARMACEUTICAL RESEARCH INSTITUTE
Princeton, NJ (in Seattle, WA from 1990-1997)

1990 - 2003

Associate Director, Immunology and Oncology Drug Discovery (1999 - 2003)

Directed a research group including 25 scientists (Ph.D. level and research associates) to identify, validate and develop novel targets for therapeutic intervention in both immunological/inflammatory diseases and cancer. Managed annual research budget for group (\$500K) for laboratory operations, travel and training of scientific staff. Senior leader guiding the direction of the research effort in all pre-clinical drug discovery phases, including administrative functions, reporting to the Vice President of Immunology and Oncology Drug Discovery.

- Established a research group to identify novel targets for therapeutic intervention in both immunological/inflammatory diseases and cancer. Group developed reagents, assays, screens and analyses on over fifteen targets for future drug discovery projects
- Validated novel targets through bioinformatics, microarray technologies, Taqman for expression profiling, transgenic mouse development and analysis, flow cytometry, full-length cloning, monoclonal antibody generation and general protein expression/purification
- Generated eight new screening assays (enzymes, protein-protein interactions, receptor systems) in 1.5 years with reduced cycle time (3-6 month turnaround time) from target validation to screening campaign
- Transitioned an early-phase project on Itk kinase to full-phase status in 1999 (screening campaign, lead identification, followed by significant chemistry support for SAR), taking a small molecule inhibitor to preclinical animal model testing stages and identifying efficacious compounds
- In-licensed a project on p38 from an external partner at an early phase, then transitioned it to full-phase status (1999). Developed a small molecule drug candidate (2001) for IND toxicology and phase I study
- Developed a Src kinase project (1997-2000) in immunology before transitioning program to Oncology, with discovery of an optimized small molecule currently ready for phase I studies
- Co-chaired the Exelixis Oncology alliance, established to identify new targets for cancer. Nine new targets for oncology were identified in 1.5 years, and three high throughput assays were established
- Served on immunology/inflammation licensing team for identifying outside opportunities, and served on pulmonary licensing team and subcommittees for early-stage external technologies. Efforts led to the in-licensing of the p38 project and licenses for using inflammatory target technologies

Principal Scientist, Immunological Diseases (1997 - 1999)

Established a Signal Transduction group to identify small molecule therapeutics to treat immunological and inflammatory disorders. Group included 4 Ph.D. level investigators and 11 associate scientists involved in projects relating to targeting intracellular signaling components for identification of new drug candidates

Senior Research Investigator II, Immunodeficiency and Immunomodulation (1993 - 1997)

Seattle, WA (former Oncogen biotechnology company purchased by Bristol-Myers Squibb Company)

Senior Research Investigator I, Immunodeficiency and Immunomodulation (1990 - 1993)

Seattle, WA (former Oncogen biotechnology company purchased by Bristol-Myers Squibb Company)

UNIVERSITY OF VIRGINIA, DEPARTMENT OF MICROBIOLOGY AND CANCER CENTER **1986 - 1990**
Charlottesville, VA

Postdoctoral fellow, Oncology (advisor: J. Thomas Parsons, Ph.D.)

- Identified novel mechanisms of p60^{src} activation in carcinogen-transformed embryonic cells
- Discovered novel tyrosine-phosphorylated substrates of the p60^{src} oncogene by monoclonal antibody generation and biochemical characterization
- Commercialized monoclonal antibodies to FAK, tensin, p120^{cas}, pp60^{src}, phosphotyrosine and cortactin

EDUCATION

Ph.D. University of Miami (Immunology and Microbiology)

B.A. University of California, Berkeley (Genetics)

HONORS, AWARDS, SCHOLARSHIPS AND FELLOWSHIPS

Bristol-Myers Squibb Excellence Awards	1996 - 2002
NIH Postdoctoral Fellowship Grant (F32-CA08316), University of Virginia	1987 - 1990
Presidential Scholarship, University of Miami	1981 - 1986
Honors Society, University of California, Berkeley	1979 - 1980

PROFESSIONAL AFFILIATIONS

American Association for Cancer Research
American Association of Immunologists
American Society for Microbiology
American Association for the Advancement of Science

AD HOC EDITORIAL ACTIVITY

Journal of Immunology	Molecular and Cellular Biology
Jl: Cutting Edge	Oncogene
Journal of Clinical Investigation	Journal of Cellular Physiology
Journal of Biological Chemistry	Antiviral Chemistry & Chemotherapy
Proc. Natl. Acad. Sci. USA	Blood

SELECTED INVITED PRESENTATIONS

Regulated association between the SH3 domain of the Emt/Itk tyrosine kinase and multiple intracellular ligands. Lymphocyte Signal Transduction Workshop, Santorini, Greece (October, 2000)

Signal transduction through the T-lymphocyte receptors CD2 and LFA-1. Sugan, South San Francisco, California (June, 1996)

Lymphocyte antigen receptor activation of a novel FAK-related tyrosine kinase substrate. Lymphocyte Activation Meeting, Keystone Symposia on Molecular and Cellular Biology, Keystone, Colorado (April, 1994)

T-cell signaling via integrin receptors and immunoglobulin-superfamily molecules. University of Chicago, Committee on Immunology Seminar Series, Chicago, Illinois (March, 1994)

T-cell signaling through integrins and Ig superfamily receptors. Seattle Biomedical Research Institute, Seminar Series, Seattle, Washington (March, 1993)

β_2 -integrin signaling in T-cells through PLC γ 1 is TCR-dependent. Keystone Symposium on Phosphorylation/Dephosphorylation in Signal Transduction, Keystone, Colorado (January, 1993)

Regulation of TCR-induced PLC γ 1 tyrosine phosphorylation by CD45. Plenary seminar at Biochemical Immunology Group Colloquium on the Structure and Function of the Leukocyte Common Antigen CD45, Edinburgh, Scotland (September, 1991)

PATENTS AND INVENTIONS

Raitano, A., S. B. Kanner, P. Challita, J. J. Perez-Villar, W. Ge, and A. Jakobovits. Nucleic acids and corresponding proteins entitled 158P3D2 useful in treatment and detection of cancer. November, 2004

Kanner, S. B., A. Raitano, P. Challita, J. J. Perez-Villar, W. Ge, and A. Jakobovits. Nucleic acids and corresponding proteins entitled 58P1D12 useful in treatment and detection of cancer. US-20050136435-A1; August, 2004

Raitano, A., P. Challita, J. J. Perez-Villar, W. Ge, S. B. Kanner and A. Jakobovits. Nucleic acids and corresponding proteins entitled 109P1D4 useful in treatment and detection of cancer. WO-04098515-A2; April, 2004

Barrish, J. C., J. Das, S. B. Kanner, C. Liu, S. H. Spergel, J. Wityak, A. M. P. Doweyko, and J. A. Furch. Thiazolyl inhibitors of Tec family tyrosine kinases. US6706717; March, 2004

Jakobovits, A., R. K. Morrison, A. B. Raitano, P. M. Challita-Eid, J. J. Perez-Villar, K. J. M. Morrison, M. Faris, W. Ge, J. Gudas and S. B. Kanner. Nucleic acid and corresponding protein named 158P1D7 useful in the treatment and detection of bladder and other cancers. WO-04072263-A2; February, 2004

Kanner, S. B., A. Raitano, A. Jakobovits, P. Challita-Eid, W. Ge, J. J. Perez-Villar and M. Faris. Nucleic acids and corresponding proteins entitled 254P1D6B useful in treatment and detection of cancer. US-2004-0214212-A1; January, 2004

Perez-Villar, J. J., H. Chang, W-P. Yang, Y. Wu, G. S. Whitney and S. B. Kanner. Identification and cloning of a full-length human Clnk-related gene, MIST (Mast Cell Immunoreceptor Signal Transducer). US-020155563-A1; October, 2002

Chang, H., W-P. Yang, Y. Wu, G. S. Whitney, J. J. Perez-Villar and S. B. Kanner. Cloning and expression of human SLAP-2: a novel SH2/SH3 domain-containing human SLAP homologue having immune cell-specific expression. WO-0242457-A1; May, 2002

Kanner, S. B., A. B. Reynolds, S. J. Parsons and J. T. Parsons. Monoclonal antibodies to p125^{FAK}, p120^{cm}, cortactin, pp60^{src} and tensin. Licensed and commercialized from the University of Virginia to Upstate/Cell Signaling Solutions; 1991

Kanner, S. B., A. B. Reynolds and J. T. Parsons. Monoclonal antibody 6G9 to phosphotyrosine. Licensed and commercialized from the University of Virginia to Covance, Inc./Berkeley Antibody Company; 1991

PUBLICATIONS

1. Parks, W. P., G. B. Scott, **S. B. Kanner**, E. S. Hubbell, M. A. Fischl, G. M. Dickinson and E. R. Schiff. (1984) Acquired immunodeficiency syndrome and human T-cell leukemia virus in Miami: a household approach. *In* Human T-cell Leukemia Viruses (R. C. Gallo, M. Essex, and L. Gross, eds.), Cold Spring Harbor Press, New York. pp. 381-391
2. **Kanner, S. B.**, C. Cheng-Mayer, R. B. Geffin, W. P. Parks, G. A. Beltz, L. O. Arthur, K. P. Samuel and T. S. Papas. (1986) Human retroviral *env* and *gag* polypeptides: serologic assays to measure infection. *J. Immunol.* 137:674-678
3. **Kanner, S. B.**, E. S. Parks, G. B. Scott and W. P. Parks. (1987) Simultaneous infections with human T cell leukemia virus type I and the human immunodeficiency virus. *J. Inf. Dis.* 155:617-625
4. **Kanner, S. B.**, S. J. Parsons, J. T. Parsons and T. M. Gilmer. (1988) Activation of pp60^{c-src} tyrosine kinase specific activity in tumor-derived Syrian hamster embryo cells. *Oncogene* 2:327-335
5. Reynolds, A. B., D. J. Roesel, **S. B. Kanner** and J. T. Parsons. (1989) Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular *src* gene. *Mol. Cell. Biol.* 9:629-638
6. **Kanner, S. B.**, T. M. Gilmer, A. B. Reynolds and J. T. Parsons. (1989) Novel tyrosine phosphorylations accompany the activation of pp60^{c-src} during chemical carcinogenesis. *Oncogene* 4:295-300
7. **Kanner, S. B.**, A. B. Reynolds and J. T. Parsons. (1989) Immunoaffinity purification of tyrosine-phosphorylated cellular proteins. *J. Immunol. Methods* 120:115-124
8. Reynolds, A. B., **S. B. Kanner**, H-C. R. Wang and J. T. Parsons. (1989) Stable association of activated pp60^{src} with two tyrosine-phosphorylated cellular proteins. *Mol. Cell. Biol.* 9:3951-3958
9. Ely, C. M., K. M. Oddie, J. S. Litz, A. J. Rossomando, **S. B. Kanner**, T. W. Sturgill, and S. J. Parsons. (1990) A 42 kD tyrosine kinase substrate linked to chromaffin cell secretion exhibits an associated MAP kinase activity and is highly related to a 42 kD mitogen-stimulated protein in fibroblasts. *J. Cell Biol.* 110:731-742
10. **Kanner, S. B.**, A. B. Reynolds, R. R. Vines and J. T. Parsons. (1990) Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc. Natl. Acad. Sci. USA* 87:3328-3332
11. **Kanner, S. B.**, A. B. Reynolds and J. T. Parsons. (1991) Tyrosine phosphorylation of a 120-kDa pp60^{src} substrate upon epidermal growth factor and platelet-derived growth factor receptor stimulation and in polyomavirus middle-T-antigen-transformed cells. *Mol. Cell. Biol.* 11:713-720
12. Bouton, A. H., **S. B. Kanner**, R. R. Vines, H-C. R. Wang, J. B. Gibbs and J. T. Parsons. (1991) Transformation by pp60^{src} or stimulation of cells with epidermal growth factor induces the stable association of tyrosine phosphorylated cellular proteins with GTPase activating protein. *Mol. Cell. Biol.* 11:945-953

13. Bouton, A. H., **S. B. Kanner**, R. R. Vines and J. T. Parsons. (1991) Tyrosine phosphorylation of three cellular proteins correlates with transformation of rat 1 cells by pp60^{src}. *Mol. Carcinogenesis* 4:145-152
14. **Kanner, S. B.**, A. B. Reynolds, H-C. R. Wang, R. R. Vines and J. T. Parsons. (1991) The SH2 and SH3 domains of pp60^{src} direct stable association with tyrosine-phosphorylated proteins p130 and p110. *EMBO J.* 10:1689-1698
15. Wu, H., A. B. Reynolds, **S. B. Kanner**, R. R. Vines and J. T. Parsons. (1991) Identification and characterization of a novel cytoskeleton-associated pp60^{src} substrate. *Mol. Cell. Biol.* 11:5113-5124
16. Ledbetter, J. A., **S. B. Kanner**, G. L. Schieven and J. Deans. (1991) Transmembrane signals in T cells: tyrosine phosphorylation regulates phospholipase C activation. In *Signaling Mechanisms in Secretory and Immune Cells* (J. R. Martinez, B. S. Edwards and J. C. Seagrave, eds.), San Francisco Press, California. pp. 51-56
17. **Kanner, S. B.**, T. J. Kavanagh, A. Grossmann, S-L. Hu, J. B. Bolen, P. S. Rabinovitch and J. A. Ledbetter. (1992) Sulfhydryl oxidation down-regulates T-cell signaling and inhibits tyrosine phosphorylation of phospholipase C γ 1. *Proc. Natl. Acad. Sci. USA* 89:300-304
18. **Kanner, S. B.** and J. A. Ledbetter. (1992) CD45 regulates TCR-induced signalling through tyrosine phosphorylation of phospholipase C γ 1. *Biochem. Soc. Trans.* 20:178-184
19. Turka, L. A., **S. B. Kanner**, G. L. Schieven, C. B. Thompson and J. A. Ledbetter. (1992) CD45 modulates T cell receptor/CD3-induced activation of human thymocytes via regulation of tyrosine phosphorylation. *Eur. J. Immunol.* 22:551-557
20. **Kanner, S. B.**, J. P. Deans and J. A. Ledbetter. (1992) Regulation of CD3-induced phospholipase C- γ 1 (PLC γ 1) tyrosine phosphorylation by CD4 and CD45 receptors. *Immunology* 75:441-447
21. Deans, J. P., **S. B. Kanner**, R. M. Torres and J. A. Ledbetter. (1992) Interaction of CD4:lck with the T cell receptor/CD3 complex induces early signaling events in the absence of CD45 tyrosine phosphatase. *Eur. J. Immunol.* 22:661-668
22. **Kanner, S. B.**, N. K. Damle, J. Blake, A. Aruffo and J. A. Ledbetter. (1992) CD2/LFA-3 ligation induces phospholipase-C γ 1 tyrosine phosphorylation and regulates CD3 signaling. *J. Immunol.* 148:2023-2029
23. Sancho, J., J. A. Ledbetter, M-S. Choi, **S. B. Kanner**, J. P. Deans and C. Terhorst. (1992) CD3- ζ surface expression is required for CD4-p56^{lck}-mediated up-regulation of T cell antigen receptor-CD3 signaling in T cells. *J. Biol. Chem.* 267:7871-7879
24. Gilliland, L. K., G. L. Schieven, N. A. Norris, **S. B. Kanner**, A. Aruffo and J. A. Ledbetter. (1992) Lymphocyte lineage-restricted tyrosine-phosphorylated proteins that bind PLC γ 1 SH2 domains. *J. Biol. Chem.* 267:13610-13616

25. Aruffo, A., **S. B. Kanner**, D. Sgroi, J. A. Ledbetter and I. Stamenkovic. (1992) CD22-mediated stimulation of T cells regulates TCR/CD3-induced signaling. *Proc. Natl. Acad. Sci. USA* **89**:10242-10246
26. **Kanner, S. B.**, N. Ødum, L. Grosmaire, S. Masewicz, A. Svejgaard and J. A. Ledbetter. (1992) Superantigen and HLA-DR ligation induce phospholipase-C γ 1 activation in class II⁺ T-cells. *J. Immunol.* **148**:3482-3488
27. Kavanagh, T. J., A. Grossmann, J. C. Jinneman, **S. B. Kanner**, C. C. White, D. L. Eaton, J. A. Ledbetter and P. S. Rabinovitch. (1993) The effect of 1-chloro-2, 4-dinitrobenzene exposure on antigen receptor (CD3)-stimulated transmembrane signal transduction in purified subsets of human peripheral blood lymphocytes. *Toxicol. Appl. Pharmacol.* **119**:91-99
28. Ledbetter, J. A., J. P. Deans, A. Aruffo, L. S. Grosmaire, **S. B. Kanner**, J. B. Bolen and G. L. Schieven. (1993) CD4, CD8 and the role of CD45 in T-cell activation. *Curr. Opin. Immunol.* **5**:334-340
29. Ødum, N., **S. B. Kanner**, J. A. Ledbetter and A. Svejgaard. (1993) MHC class II molecules deliver costimulatory signals in human T cells through a functional linkage with IL-2-receptors. *J. Immunol.* **150**:5289-5298
30. Chalupny, N. J., **S. B. Kanner**, G. L. Schieven, S. Wee, L. K. Gilliland, A. Aruffo and J. A. Ledbetter. (1993) Tyrosine phosphorylation of CD19 in pre-B and mature B cells. *EMBO J.* **12**:2691-2696
31. **Kanner, S. B.**, L. S. Grosmaire, J. A. Ledbetter and N. K. Damle. (1993) β_2 -integrin LFA-1 signaling through phospholipase-C γ 1 activation. *Proc. Natl. Acad. Sci. USA* **90**:7099-7103
32. Whitney, G. S., P-Y. Chan, J. Blake, W. L. Cosand, M. G. Neubauer, A. Aruffo and **S. B. Kanner**. (1993) Human T and B lymphocytes express a structurally conserved focal adhesion kinase (pp125^{FAK}). *DNA Cell Biol.* **12**:823-830
33. Parsons, M., J. A. Ledbetter, G. L. Schieven, A. E. Nel and **S. B. Kanner**. (1994) Developmental regulation of pp44/46, tyrosine-phosphorylated proteins associated with tyrosine/serine kinase activity in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **63**:69-78
34. Chan, P-Y., **S. B. Kanner**, G. S. Whitney and A. Aruffo. (1994) A transmembrane-anchored chimeric focal adhesion kinase is constitutively activated and phosphorylated at tyrosine residues identical to pp125^{FAK}. *J. Biol. Chem.* **269**:20567-20574
35. Schieven, G. L., R. S. Mittler, S. G. Nadler, J. M. Kiriara, J. B. Bolen, **S. B. Kanner** and J. A. Ledbetter. (1994) ZAP-70 tyrosine kinase, CD45, and T cell receptor involvement in UV- and H₂O₂-induced T cell signal transduction. *J. Biol. Chem.* **269**:20718-20726
36. Dietsch, M. T., P-Y. Chan, **S. B. Kanner**, L. K. Gilliland, J. A. Ledbetter, P. S. Linsley and A. Aruffo. (1994) Coengagement of CD2 with LFA-1 or VLA-4 by bispecific ligand fusion proteins primes T cells to respond more effectively to T cell receptor-dependent signals. *J. Leukoc. Biol.* **56**:444-452

37. **Kanner, S. B.**, A. Aruffo and P-Y. Chan. (1994) Lymphocyte antigen receptor activation of a focal adhesion kinase-related tyrosine kinase substrate. *Proc. Natl. Acad. Sci. USA* **91**:10484-10487
38. Wong, S. C.-T., **S. B. Kanner**, J. A. Ledbetter, S. Gupta, G. Kumar and A. E. Nel. (1995) Evidence for LFA-1/ICAM-1 dependent stimulation of protein tyrosine phosphorylation in human B lymphoid cell lines during homotypic adhesion. *J. Leukoc. Biol.* **57**:343-351
39. **Kanner, S. B.** and O. K. Haffar. (1995) HIV-1 down-regulates CD4 costimulation of TCR/CD3-directed tyrosine phosphorylation through CD4/p56^{lck} dissociation. *J. Immunol.* **154**:2996-3005
40. Ohnishi, H., J. A. Ledbetter, **S. B. Kanner**, P. S. Linsley, T. Tanaka, A. M. Geller and M. Kotb. (1995) CD28 cross-linking augments TCR-mediated signals and costimulates superantigen responses. *J. Immunol.* **154**:3180-3193
41. Grossmann, A., P. S. Rabinovitch, T. J. Kavanagh, J. C. Jinneman, L. K. Gilliland, J. A. Ledbetter and **S. B. Kanner**. (1995) Activation of murine T-cells via phospholipase-C γ 1-associated protein tyrosine phosphorylation is reduced with aging. *J. Gerontol.* **50A**:B205-B212
42. Tarakhovsky, A., **S. B. Kanner**, J. Hombach, J. A. Ledbetter, W. Müller, N. Killeen and K. Rajewsky. (1995) A role for CD5 in TCR-mediated signal transduction and thymocyte selection. *Science* **269**:535-537
43. Nel, A. E., S. Gupta, L. Lee, J. A. Ledbetter and **S. B. Kanner**. (1995) Ligation of the T-cell antigen receptor (TCR) induces association of hSos1, ZAP-70, phospholipase C- γ 1, and other phosphoproteins with Grb2 and the ζ -chain of the TCR. *J. Biol. Chem.* **270**:18428-18436
44. **Kanner, S. B.**, L. S. Grosmaire, J. Blake, G. L. Schieven, S. Masewicz, N. Ødum and J. A. Ledbetter. (1995) ZAP-70 and p72^{syk} are signaling response elements through MHC class II molecules. *Tissue Antigens* **46**:145-154
45. Das, A., G. C. Peterson, **S. B. Kanner**, U. Frevert and M. Parsons. (1996) A major tyrosine-phosphorylated protein of *Trypanosoma brucei* is a nucleolar RNA-binding protein. *J. Biol. Chem.* **271**:15675-15681
46. **Kanner, S. B.** (1996) Focal adhesion kinase-related fakB is regulated by the integrin LFA-1 and interacts with the SH3 domain of phospholipase C γ 1. *Cell. Immunol.* **171**:164-169
47. Ochs, H. D., Q. Zhu, T. Liu, C. Watanabe, **S. B. Kanner**, D. Hollenbaugh and A. Aruffo. (1996) Wiskott-Aldrich syndrome: The WAS protein, phenotype and genotype. *In Progress in Immunodeficiency VI* (A. Fasth and J. Bjorkander, eds.), Elsevier Science B.V., Goteborg, Sweden. pp. 85-96
48. Kotb, M., **S. B. Kanner** and J. A. Ledbetter. (1997) Biochemical analysis of TCR mediated signaling. *In The Human Antigen T-cell Receptor: Selected Protocols and Applications* (J. R. Oksenberg, ed.), R.G. Landes Company and Chapman & Hall, Texas. pp. 489-545

49. Ng, T. T. C., **S. B. Kanner**, M. J. Humphries, R. G. Wickremasinghe, K. E. Nye, J. Anderson, S. H. Khoo and W. J. W. Morrow. (1997) The integrin-triggered rescue of T lymphocyte apoptosis is blocked in HIV-1-infected individuals. *J. Immunol.* **158**:2984-2999
50. Scholler, J. K. and **S. B. Kanner**. (1997) The human p167 gene encodes a unique structural protein that contains centrosomin A homology and associates with a multicomponent complex. *DNA Cell Biol.* **16**:515-531
51. Lansing, T. J., B. F. Turk, **S. B. Kanner** and T. M. Gilmer. (1997) Mutational activation of pp60^{c-src} leads to a tumorigenic phenotype in a preneoplastic Syrian hamster embryo cell line. *Cancer Res.* **57**:1962-1969
52. Zhu, Q., C. Watanabe, T. Liu, D. Hollenbaugh, R. M. Blaese, **S. B. Kanner**, A. Aruffo and H. D. Ochs. (1997) The Wiscott-Aldrich syndrome/X-linked thrombocytopenia: WASP gene mutations, protein expression, and phenotype. *Blood* **90**:2680-2689
53. Brockdorff, J., **S. B. Kanner**, M. Nielsen, N. Borregaard, C. Geisler, A. Svejgaard and N. Ødum. (1998) Interleukin-2 induces β 2-integrin-dependent signal transduction involving the focal adhesion kinase-related protein B (fakB). *Proc. Natl. Acad. Sci. USA* **95**:6959-6964
54. Loo, D. T., **S. B. Kanner** and A. Aruffo. (1998) Filamin binds to the cytoplasmic domain of the β 1-integrin: identification of amino acids responsible for this interaction. *J. Biol. Chem.* **273**:23304-23312
55. Connelly, R. J., M. S. Hayden, J. K. Scholler, T. T. Tsu, B. Dupont, J. A. Ledbetter and **S. B. Kanner**. (1998) Mitogenic properties of a bispecific single chain Fv-Ig fusion generated from CD2-specific mAb to distinct epitopes. *Int. Immunol.* **10**:1863-1872
56. Ng, T. T. C., I. E. Collins, **S. B. Kanner**, M. J. Humphries, N. Amft, R. G. Wickremasinghe, D. D'Cruz, K. E. Nye and W. J. W. Morrow. (1999) Integrin signalling defects in T-lymphocytes in systemic lupus erythematosus. *Lupus* **8**:39-51
57. Perez-Villar, J. J., G. S. Whitney, M. A. Bowen, D. H. Hewgill, A. A. Aruffo and **S. B. Kanner**. (1999) CD5 negatively regulates the T-cell antigen receptor signal transduction pathway: involvement of SH2-containing phosphotyrosine phosphatase SHP-1. *Mol. Cell. Biol.* **19**:2903-2912
58. Perez-Villar, J. J. and **S. B. Kanner**. (1999) Regulated association between the tyrosine kinase Emt/Itk/Tsk and phospholipase-C γ 1 in human T lymphocytes. *J. Immunol.* **163**:6435-6441
59. Scholler, J. K., J. J. Perez-Villar, K. O'Day and **S. B. Kanner**. (2000) Engagement of the T-lymphocyte antigen receptor regulates association of son-of-sevenless homologues with the SH3 domain of phospholipase C γ 1. *Eur. J. Immunol.* **30**:2378-2387
60. van Seventer, G. A., H. J. Salmen, S. F. Law, G. M. O'Neill, M. M. Mullen, A. M. Franz, **S. B. Kanner**, E. A. Golemis and J. M. van Seventer. (2001) Focal adhesion kinase regulates β 1 integrin-dependent T cell migration through an HEF1 effector pathway. *Eur. J. Immunol.* **31**:1417-1427

61. Perez-Villar, J. J., K. O'Day, D. H. Hewgill, S. G. Nadler and **S. B. Kanner**. (2001) Nuclear localization of the tyrosine kinase Itk and interaction of its SH3 domain with karyopherin α (Rch1 α). *Int. Immunol.* **13**:1265-1274
62. Perez-Villar, J. J., G. S. Whitney, M. T. Sitnick, R. J. Dunn, S. Venkatesan, K. O'Day, G. L. Schieven, T. Lin and **S. B. Kanner**. (2002) Phosphorylation of the linker for activation of T-cells by Itk promotes recruitment of Vav. *Biochemistry* **41**:10732-10740
63. **Kanner, S. B.** and J. J. Perez-Villar. (2003) Altering T cell activation by targeting the multi-domain tyrosine kinase Itk. *Trends Immunol.* **24**:249-253
64. Wityak, J., J. Das, R. V. Moquin, Z. Shen, J. Lin, P. Chen, A. M. Doweyko, S. Pitt, S. Pang, D. R. Shen, Q. Fang, H. F. de Fex, G. L. Schieven, **S. B. Kanner** and J. C. Barrish. (2003) Discovery and initial SAR of 2-amino-5-carboxamidothiazoles as inhibitors of the Src-family kinase p56^{Lck}. *Bioorg. Med. Chem. Lett.* **13**:4007-4010
65. Chen, P., A. M. Doweyko, D. Norris, H. H. Gu, S. H. Spergel, J. Das, R. V. Moquin, J. Lin, J. Wityak, E. J. Iwanowicz, K. W. McIntyre, D. J. Shuster, K. Behnia, S. Chong, H. de Fex, S. Pang, S. Pitt, D. R. Shen, S. Thrall, P. Stanley, O. R. Kocy, M. R. Witmer, **S. B. Kanner**, G. L. Schieven and J. C. Barrish. (2004) Imidazoquinoxaline Src-family kinase p56Lck inhibitors: SAR, QSAR, and the discovery of (*S*)-*N*-(2-chloro-6-methylphenyl)-2-(3-methyl-1-piperazinyl)imidazo-[1,5-*a*]pyrido[3,2-*e*]pyrazin-6-amine (BMS-279700) as a potent and orally active inhibitor with excellent in vivo anti-inflammatory activity. *J. Med. Chem.* **47**:4517-4529
66. Lin, T., K. W. McIntyre, J. Das, C. Liu, K. D. O'Day, B. Penhallow, C-Y. Hung, D. J. Shuster, X. Yang, R. Townsend, J. Postelnek, S. H. Spergel, J. Lin, R. V. Moquin, J. A. Furch, A. V. Kamath, H. Zhang, P. H. Marathe, J. J. Perez-Villar, A. Doweyko, L. Killar, J. H. Dodd, J. C. Barrish, J. Wityak and **S. B. Kanner**. (2004) Selective Itk inhibitors block T-cell activation and murine lung inflammation. *Biochemistry* **43**:11056-11062
67. Leftheris, K., G. Ahmed, R. Chan, A. J. Dyckman, Z. Hussain, K. Ho, J. Hynes, Jr., J. Letourneau, W. Li, S. Lin, A. Metzger, K. J. Moriarty, C. Riviello, Y. Shimshock, J. Wen, J. Wityak, S. T. Wroblewski, H. Wu, J. Wu, M. Desai, K. M. Gillooly, T. H. Lin, D. Loo, K. W. McIntyre, S. Pitt, D. R. Shen, S. J. Shuster, R. Zhang, D. Diller, A. Doweyko, J. Sack, J. Baldwin, J. Barrish, J. Dodd, I. Henderson, **S. B. Kanner**, G. L. Schieven and M. Webb. (2004) The discovery of orally active triaminotriazine aniline amides as inhibitors of p38 MAP kinase. *J. Med. Chem.* **47**:6283-6291

Investor Street

2005
2nd Quarter

Genentech
IN BUSINESS FOR LIFE

PROFILE

Genentech (NYSE:DNA) is among the world's leading biotechnology companies. Our focus is on developing innovative therapies that address significant unmet medical needs and positively impact the world of human health. Genentech manufactures and commercializes multiple products in the United States for a variety of medical conditions, including cancer, heart attack, stroke, growth hormone deficiency, cystic fibrosis, allergic asthma and plaque psoriasis.

Herceptin[®] Trastuzumab

anti-HER² monoclonal antibody

Results from the joint interim analysis of the NSABP/NCCTG Phase III studies of Herceptin in early stage breast cancer showed that women with HER2+ breast cancer receiving Herceptin plus chemotherapy had a 52 percent reduction in the risk of disease recurrence. Preliminary survival data showed a 49 percent improvement in overall survival. Survival data continue to mature. Serious or life-threatening cardiac events, most commonly congestive heart failure occurred approximately 3 to 4 percent more often in the Herceptin plus chemotherapy arms than in the chemotherapy alone arms. Patients in these studies will continue to be followed.

MARKET DATA (AS OF 07/15/05)

Closing Price: \$89.58

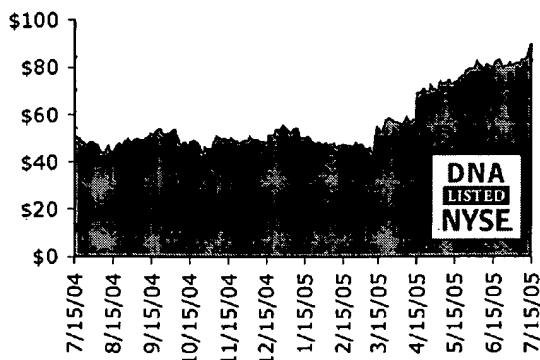
52-week Range: \$41.00-\$90.57

Market Cap: \$95.2 billion

Total Shares Outstanding: 1,062.4 million

GENENTECH STOCK PRICE

July 15, 2004 - July 15, 2005



Q2 2005 / EARLY Q3 2005 NEWS/EVENTS

- Diluted GAAP earnings per share for the second quarter of 2005 increased 69 percent to \$0.27 per share from \$0.16 per share in the second quarter of 2004. Non-GAAP earnings per share for the second quarter of 2005 increased 58 percent to \$0.30 per share from \$0.19 per share in the second quarter of 2004.
Genentech's non-GAAP financial results exclude litigation-related special charges of \$19.5 million in the second quarter of 2005 and \$13.5 million in the second quarter of 2004; and recurring charges related to the 1999 redemption of Genentech's Special Common Stock of \$34.5 million in the second quarter of 2005 and \$38.2 million in the second quarter of 2004, net of related taxes.
- Total product sales reached \$1,274.1 million for the second quarter of 2005, a 39 percent increase over product sales of \$913.4 million in the second quarter of 2004. Total product sales were comprised of U.S. product sales of \$1,215.9 million and product sales to collaborators of \$58.2 million.
- Genentech announced positive Phase III data results for Avastin, Herceptin, Lucentis and Rituxan demonstrating clinically and statistically significant outcomes: Avastin plus paclitaxel and carboplatin chemotherapies in first-line non-squamous, non-small cell lung cancer; Rituxan in patients with rheumatoid arthritis who inadequately respond to anti-TNF alpha therapies; Avastin plus paclitaxel chemotherapy in first-line metastatic breast cancer; Herceptin plus chemotherapy in early-stage breast cancer, and Lucentis in patients with minimally classic or occult wet age-related macular degeneration.
- Genentech and OSI Pharmaceuticals announced that OSI submitted a supplemental New Drug Application with the FDA for use of Tarceva plus gemcitabine chemotherapy for the treatment of advanced pancreatic cancer in patients who have not received any previous treatment.
- In the second quarter, Genentech began enrolling patients in several Phase III studies, including Rituxan in systemic lupus erythematosus, Avastin in prostate cancer, and combination Avastin and Tarceva in second-line non-small cell lung cancer. Enrollment also began in a Phase I study of a small molecule topical hedgehog antagonist candidate in basal cell carcinoma.
- Omnitarg as a single agent in breast and lung cancer was removed from the development pipeline. The Phase II study of Omnitarg in combination with chemotherapy for ovarian cancer continues to enroll.
- Positive Phase III results were reported with Lucentis in patients with wet age-related macular degeneration (AMD), showing for the first time in a Phase III trial, a statistically significant improvement in vision for patients in a disease that has remained chronic and progressive despite current treatment options. Additionally, the Phase I/II study of Lucentis demonstrated improved vision in patients with wet AMD when used in combination with verteporfin photodynamic therapy.
- Genentech and Boehringer Ingelheim announced that the ASSENT 4 percutaneous coronary intervention (PCI) trial, an open-label Phase IV study of single-bolus tenecteplase in combination with a planned PCI, was stopped early after an analysis showed mortality rates in those patients treated with tenecteplase plus PCI were higher than in the PCI alone arm.
- Genentech completed the acquisition of the Oceanside biologics manufacturing facility from Biogen Idec for approximately \$408 million in cash, plus approximately \$9 million in closing costs. Upon FDA licensure, the Oceanside facility will add 90,000 liters of capacity that will initially be dedicated to producing Avastin bulk drug substance.
- Genentech's Board of Directors authorized the extension of its current stock repurchase program for the repurchase of up to an additional \$2 billion of its common stock for a total of \$4 billion through June 30, 2006. The Board also amended the current repurchase program by increasing the maximum number of shares that can be repurchased from 50 million to 80 million shares.

Please refer to the press releases on our website at www.gene.com for more detailed information regarding data results and other corporate news.

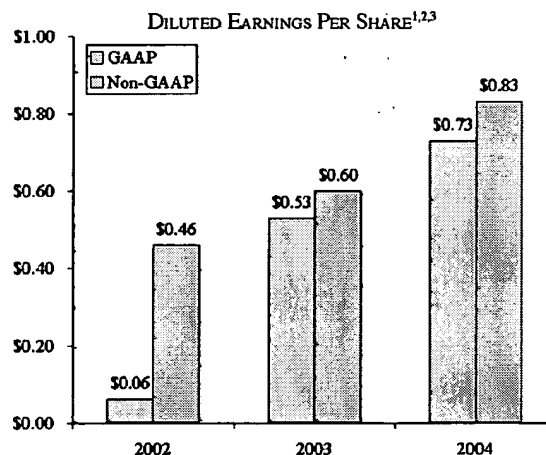
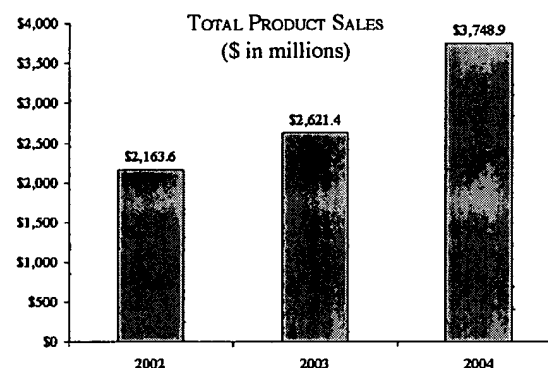
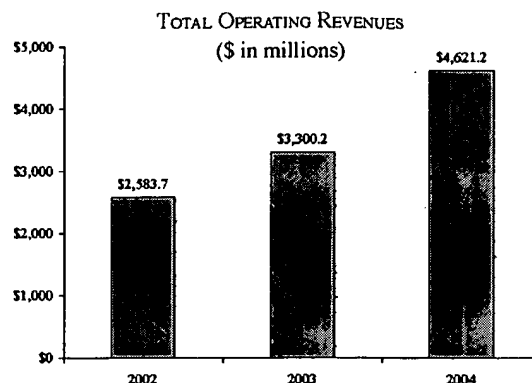
Development pipeline

AS OF JULY 15, 2005

AWAITING FDA ACTION	
TARCEVA	PANCREATIC CANCER
FDA FILING PREPARATION	
AVASTIN	METASTATIC BREAST CANCER NON-SMALL CELL LUNG CANCER SECOND-LINE COLORECTAL CANCER
HERCEPTIN	ADJUVANT BREAST CANCER FIRST-LINE METASTATIC BREAST CANCER IN COMBINATION WITH TAXOTERE
RITUXAN HEMATOLOGY/ONCOLOGY	FRONTLINE AGGRESSIVE NHL INDOLENT FRONTLINE/MAINTENANCE** NHL
RITUXAN IMMUNOLOGY	REFRACTORY RHEUMATOID ARTHRITIS
PHASE III	
AVASTIN	ADJUVANT COLORECTAL CANCER FIRST-LINE OVARIAN CANCER* PANCREATIC CANCER PROSTATE CANCER RENAL CELL CARCINOMA
LUCENTIS	WET AGE-RELATED MACULAR DEGENERATION
RITUXAN HEMATOLOGY/ONCOLOGY	RELAPSED, CHRONIC LYMPHOCYTIC LEUKEMIA
RITUXAN IMMUNOLOGY	ANCA-ASSOCIATED VASCULITIS LUPUS NEPHRITIS* MOD-TO-SEVERE RHEUMATOID ARTHRITIS* PRIMARY PROGRESSIVE MULTIPLE SCLEROSIS SYSTEMIC LUPUS ERYTHEMATOSUS
TARCEVA	ADJUVANT NON-SMALL CELL LUNG CANCER*
TARCEVA +/- AVASTIN	SECOND-LINE NON-SMALL CELL LUNG CANCER
XOLAIR	PEDIATRIC ASTHMA
PHASE II	
2ND GENERATION ANTI-CD20	RHEUMATOID ARTHRITIS
AVASTIN	REFRACTORY OVARIAN CANCER
AVASTIN +/- TARCEVA	NON-SMALL CELL LUNG CANCER RENAL CELL CARCINOMA
OMNITARG	OVARIAN CANCER
RAPTIVA	ADULT ATOPIC DERMATITIS*
RITUXAN-IMMUNOLOGY	RELAPSED REMITTING MULTIPLE SCLEROSIS
TOPICAL VEGF	DIABETIC FOOT ULCERS*
XOLAIR	PEANUT ALLERGY
PHASE I	
ANTI-NGF	ACUTE AND CHRONIC PAIN
APO2L/TRAIL	CANCER THERAPY
BR3-Fc	RHEUMATOID ARTHRITIS
TOPICAL HEDGEHOG ANTAGONIST	BASAL CELL CARCINOMA

*PREPARING FOR PHASE

**ONE AREA OF CONCERN FOR FDA IS THE USE OF THE TERM "MAINTENANCE", SO WE WILL BE WORKING TOWARD A NOMENCLATURE THAT FDA FEELS BETTER DESCRIBES THIS APPROACH.



(1) Non-GAAP amounts exclude: (i) recurring charges related to the 1999 redemption of our Special Common Stock of \$145.5 million in 2004, \$154.3 million in 2003, \$155.7 million in 2002; (ii) litigation settlements (net of litigation charges) of \$113.1 million in 2003, and litigation charges (net of a released accrual) of \$37.1 million in 2004 and \$543.9 million in 2002; (iii) a \$47.6 million charge, net of tax, in 2003 as a cumulative effect of the change in accounting principle for our adoption of the Financial Accounting Standards Board (or FASB) Interpretation No. 46, "Consolidation of Variable Interest Entities" on July 1, 2003.

(2) Net income in 2002 also reflects our adoption of FAS 141, "Business Combinations" and 142, "Goodwill and Other Intangible Assets" on January 1, 2002. As a result of our adoption, reported net income increased by approximately \$157.6 million (or \$0.15 per share) due to the cessation of goodwill amortization and the amortization of our trained and assembled workforce intangible asset.

(3) All share and per share amounts reflect the May 2004 two-for-one stock split of our Common Stock.

INVESTOR CONTACT:
Diane Schrick — Manager, Investor Relations
Genentech, Inc.
One DNA Way, South San Francisco, CA 94080
Phone: 650-225-1599; Fax: 650-225-8326
Web: www.gene.com
Email: investor.relations@gene.com

©2005 Genentech, Inc.

Herceptin®

Trastuzumab

anti-HER2 monoclonal antibody

WARNINGS:

CARDIOMYOPATHY

HERCEPTIN administration can result in the development of ventricular dysfunction and congestive heart failure. Left ventricular function should be evaluated in all patients prior to and during treatment with HERCEPTIN. Discontinuation of HERCEPTIN treatment should be strongly considered in patients who develop a clinically significant decrease in left ventricular function. The incidence and severity of cardiac dysfunction was particularly high in patients who received HERCEPTIN in combination with anthracyclines and cyclophosphamide. (See WARNINGS.)

HYPERSENSITIVITY REACTIONS INCLUDING ANAPHYLAXIS

INFUSION REACTIONS

PULMONARY EVENTS

HERCEPTIN administration can result in severe hypersensitivity reactions (including anaphylaxis), infusion reactions, and pulmonary events. Rarely, these have been fatal. In most cases, symptoms occurred during or within 24 hours of administration of HERCEPTIN. HERCEPTIN infusion should be interrupted for patients experiencing dyspnea or clinically significant hypotension. Patients should be monitored until signs and symptoms completely resolve. Discontinuation of HERCEPTIN treatment should be strongly considered for patients who develop anaphylaxis, angioedema, or acute respiratory distress syndrome. (See WARNINGS.)

DESCRIPTION

HERCEPTIN (Trastuzumab) is a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity to a cell-based assay (Kd=5 nM) to the extracellular domain of the human epidermal growth factor receptor 2 protein, HER2.^{1,2} The antibody is an IgG₁ kappa that contains human framework regions with the complementarity-determining regions of a murine antibody (4D5) that binds to HER2.

The humanized antibody against HER2 is produced by a mammalian cell (Chinese Hamster Ovary [CHO]) suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product.

HERCEPTIN is a sterile, white to pale yellow, preservative-free lyophilized powder for intravenous (IV) administration. The nominal content of each HERCEPTIN vial is 440 mg Trastuzumab, 9.9 mg L-histidine HCl, 8.4 mg L-histidine, 400 mg α,α-trehalose dihydrate, and 1.8 mg polysorbate 20, USP. Reconstitution with 20 mL of the supplied Bacteriostatic Water for Injection (BWI), USP, containing 1.1% benzyl alcohol as a preservative, yields a multi-dose solution containing 21 mg/mL Trastuzumab, at a pH of approximately 6.

CLINICAL PHARMACOLOGY

General

The HER2 (or c-erbB2) proto-oncogene encodes a transmembrane receptor protein of 185 kDa, which is structurally related to the epidermal growth factor receptor. HER2 protein overexpression is observed in 25%-30% of primary breast cancers. HER2 protein overexpression can be determined using immunohistochemistry (IHC) and gene amplification can be determined using fluorescence in situ hybridization (FISH) of fixed tumor blocks.¹ In referenced studies where HERCEPTIN use was not studied,² approximately 96-98% of biopsy specimens that were found to have protein overexpression also had gene amplification and 100% of those with gene amplification also had protein overexpression.^{2,4} The precision of the determination of protein overexpression or gene amplification, however, may vary depending on the sensitivity and specificity of the particular assay and assay procedures used (see PRECAUTIONS). When compared to the referenced studies noted above, the correlation between detectable protein overexpression using immunohistochemistry (IHC) and detectable gene amplification using fluorescence in situ hybridization (FISH) was not as high in the studies of HERCEPTIN clinical trial specimens (see CLINICAL STUDIES: HER2 Detection and HER2 Assay Concordance Studies and PRECAUTIONS: HER2 Testing).

Trastuzumab has been shown, in both *in vitro* assays and in animals, to inhibit the proliferation of human tumor cells that overexpress HER2.^{2,4}

Trastuzumab is a mediator of antibody-dependent cellular cytotoxicity (ADCC).^{2,4} *In vitro*, HERCEPTIN-mediated ADCC has been shown to be preferentially exerted on HER2 overexpressing cancer cells compared with cancer cells that do not overexpress HER2.

Pharmacokinetics

The pharmacokinetics of Trastuzumab were studied in breast cancer patients with metastatic disease. Short duration intravenous infusions of 10 to 500 mg once weekly demonstrated dose-dependent pharmacokinetics. Mean half-life increased and clearance decreased with increasing dose level. The half-life averaged 1.7 and 12 days at the 10 and 500 mg dose levels, respectively. Trastuzumab's volume of distribution was approximately that of serum volume (44 mL/kg). At the highest weekly dose studied (500 mg), mean peak serum concentrations were 377 microgram/mL.

In studies using a loading dose of 4 mg/kg followed by a weekly maintenance dose of 2 mg/kg, a mean half-life of 5.8 days (range=1 to 32 days) was observed. Between Weeks 18 and 32, Trastuzumab serum concentrations reached a steady state with mean trough and peak concentrations of approximately 79 microgram/mL and 123 microgram/mL, respectively.

Detectable concentrations of the circulating extracellular domain of the HER2 receptor (shed antigen) are found in the sera of some patients with HER2 overexpressing tumors. Determination of shed antigen in baseline serum samples revealed that 64% (286/447) of patients had detectable shed antigen, which ranged as high as 1880 ng/mL (median=11 ng/mL). Patients with higher baseline shed antigen levels were more likely to have lower serum trough concentrations. However, with weekly dosing, most patients with elevated shed antigen levels achieved target serum concentrations of Trastuzumab by Week 6.

Data suggest that the disposition of Trastuzumab is not altered based on age or serum creatinine (up to 2.0 mg/dL). No formal interaction studies have been performed.

Mean serum trough concentrations of Trastuzumab, when administered in combination with paclitaxel, were consistently elevated approximately 1.5-fold as compared with serum concentrations of Trastuzumab used in combination with anthracycline plus cyclophosphamide. In primate studies, administration of Trastuzumab with paclitaxel resulted in a reduction in Trastuzumab clearance. Serum levels of Trastuzumab in combination with cisplatin, doxorubicin or epirubicin plus cyclophosphamide did not suggest any interactions; no formal drug interaction studies were performed.

CLINICAL STUDIES

The safety and efficacy of HERCEPTIN were studied in a randomized, controlled clinical trial in combination with chemotherapy (469 patients) and an open-label single agent clinical trial (222 patients). Both trials studied patients with metastatic breast cancer whose tumors overexpress the

HERCEPTIN® (Trastuzumab)

HER2 protein. Patients were eligible if they had 2+ or 3+ levels of overexpression (based on a 0 to 3+ scale) by immunohistochemical assessment of tumor tissue performed by a central testing lab.

A multicenter, randomized, controlled clinical trial was conducted in 469 patients with metastatic breast cancer who had not been previously treated with chemotherapy for metastatic disease.¹¹ Patients were randomized to receive chemotherapy alone or in combination with HERCEPTIN given intravenously as a 4 mg/kg loading dose followed by weekly doses of HERCEPTIN at 2 mg/kg. For those who had received prior anthracycline therapy in the adjuvant setting, chemotherapy consisted of paclitaxel (175 mg/m² over 3 hours every 21 days for at least six cycles); for all other patients, chemotherapy consisted of anthracycline plus cyclophosphamide (AC: doxorubicin 60 mg/m² or epirubicin 75 mg/m² plus 600 mg/m² cyclophosphamide every 21 days for six cycles). Compared with patients in the AC subgroups (n=281), patients in the paclitaxel subgroup (n=188) were more likely to have had the following: poor prognostic factors (premenopausal status, estrogen or progesterone receptor negative tumors, positive lymph nodes), prior therapy (adjuvant chemotherapy, myeloablative chemotherapy, radiotherapy), and a shorter disease-free interval. Sixty-five percent of patients randomized to receive chemotherapy alone in this study received HERCEPTIN at the time of disease progression as part of a separate extension study.

Compared with patients randomized to chemotherapy alone, the patients randomized to HERCEPTIN and chemotherapy experienced a significantly longer median time to disease progression, a higher overall response rate (ORR), a longer median duration of response, and a longer median survival (see Table 1). These treatment effects were observed both in patients who received HERCEPTIN plus paclitaxel and in those who received HERCEPTIN plus AC, however the magnitude of the effects was greater in the paclitaxel subgroup (see CLINICAL STUDIES: HER2 Detection).

Table 1
Phase III Clinical Efficacy in First-Line Treatment

	Combined Results HERCEPTIN + All Chemo- therapy (n = 235)		Paclitaxel Subgroup HERCEPTIN + Paclitaxel (n = 92)		AC Subgroup HERCEPTIN + AC* (n = 143)		AC (n = 138)
Primary Endpoint Time to Progression ^{a,b}							
Median (months)	7.2	4.5	6.7	2.5	7.6	5.7	
95% confidence interval	6.9, 8.2	4.3, 4.9	5.2, 9.9	2.0, 4.3	7.2, 9.1	4.6, 7.1	
p-value (log rank)	<0.0001		<0.0001		0.002		
Secondary Endpoints Overall Response Rate ^a							
Rate (percent)	45	29	38	15	50	38	
95% confidence interval	39, 51	23, 35	28, 48	8, 22	42, 58	30, 46	
p-value (χ ² -test)	<0.001		<0.001		0.10		
Duration of Response^{a,b}							
Median (months)	8.3	5.8	8.3	4.3	8.4	6.4	
25%, 75% quartile	5.5, 14.8	3.9, 8.5	5.1, 11.0	3.7, 7.4	5.8, 14.8	4.5, 8.5	
Survival Time^a							
Median Survival (months)	25.1	20.3	22.1	18.4	26.8	21.4	
95% confidence interval	22.2, 29.5	16.8, 24.2	16.9, 28.6	12.7, 24.4	23.3, 32.9	18.3, 26.6	
p-value (log rank)	0.05		0.17		0.16		

*AC = anthracycline (doxorubicin or epirubicin) and cyclophosphamide.

^aAssessed by an Independent Response Evaluation Committee.

^bKaplan-Meier Estimate.

HERCEPTIN was studied as a single agent in a multicenter, open-label, single-arm clinical trial in patients with HER2 overexpressing metastatic breast cancer who had relapsed following one or two prior chemotherapy regimens for metastatic disease. Of 222 patients enrolled, 66% had received prior adjuvant chemotherapy, 68% had received two prior chemotherapy regimens for metastatic disease, and 25% had received prior myeloablative treatment with hematopoietic rescue. Patients were treated with a loading dose of 4 mg/kg IV followed by weekly doses of HERCEPTIN at 2 mg/kg IV. The ORR (complete response + partial response), as determined by an independent Response Evaluation Committee, was 14%, with a 2% complete response rate and a 12% partial response rate. Complete responses were observed only in patients with disease limited to skin and lymph nodes (see CLINICAL STUDIES: HER2 Detection).

HER2 Detection

(See PRECAUTIONS: HER2 Testing)

Detection of HER2 protein overexpression is necessary for selection of patients appropriate for HERCEPTIN therapy (see INDICATIONS). Overexpression of HER2 by tumors was an entry criterion of the two clinical studies described above. In those studies, a research-use-only IHC assay (referred to as the Clinical Trial Assay, CTA) was used.

The commercial assays described below, HercepTest® (IHC assay) and PathVision® (FISH assay), are appropriate assays to aid in the selection of patients for HERCEPTIN therapy (see CLINICAL STUDIES: HER2 Detection: HER2 Protein Overexpression Detection Methods and HER2 Gene Amplification Detection Methods). The comparability of either assay with regard to the ability to predict clinical benefit from HERCEPTIN therapy has not been prospectively studied. In addition, the utility of either assay in patients whose tumors would score as 0 or 1+ by the Clinical Trial Assay (CTA) has not been established because patients with tumors that scored as 0 or 1+ were excluded from the clinical studies described.

HER2 Protein Overexpression Detection Methods

HER2 protein overexpression can be established by measuring expressed HER2 protein using IHC methodology. In the clinical trial studies described above, specimens were tested with the CTA and scored as 0, 1+, 2+, or 3+ indicating the strongest positivity. Only patients with 2+ or 3+ positive tumors were eligible (about 33% of those screened). Data from the randomized trial suggest that the beneficial treatment effects were largely limited to patients with the highest level of HER2 protein overexpression (3+) (see Table 2). In an exploratory analysis, the relative risk (rr) for time to progression was lower in the patients whose tumors tested as CTA 3+ (rr = 0.42 with 95% CI: 0.33, 0.54) than in those tested as CTA 2+ (rr = 0.76 with 95% CI: 0.50, 1.15). The relative risk represents the risk of progression in the HERCEPTIN plus chemotherapy arm versus the chemotherapy arm. Therefore, a lower ratio represents longer time to progression in the HERCEPTIN arm. In the single-arm study of HERCEPTIN as a single agent, the overall response rate in patients whose tumors tested as CTA 3+ was 18% while in those that tested as CTA 2+, it was 6%.

HercepTest®, another IHC assay, was assessed for concordance with the CTA (see HER2 Testing: Concordance Studies), but has not been used to assess tumor specimens from the HERCEPTIN clinical studies described above.

Herceptin®

Trastuzumab

anti-HER2 monoclonal antibody

WARNINGS:

CARDIOMYOPATHY

HERCEPTIN administration can result in the development of ventricular dysfunction and congestive heart failure. Left ventricular function should be evaluated in all patients prior to and during treatment with HERCEPTIN. Discontinuation of HERCEPTIN treatment should be strongly considered in patients who develop a clinically significant decrease in left ventricular function. The incidence and severity of cardiac dysfunction was particularly high in patients who received HERCEPTIN in combination with anthracyclines and cyclophosphamide. (See WARNINGS.)

HYPERSENSITIVITY REACTIONS INCLUDING ANAPHYLAXIS

INFUSION REACTIONS

PULMONARY EVENTS

HERCEPTIN administration can result in severe hypersensitivity reactions (including anaphylaxis), infusion reactions, and pulmonary events. Rarely, these have been fatal. In most cases, symptoms occurred during or within 24 hours of administration of HERCEPTIN. HERCEPTIN infusion should be interrupted for patients experiencing dyspnea or clinically significant hypotension. Patients should be monitored until signs and symptoms completely resolve. Discontinuation of HERCEPTIN treatment should be strongly considered for patients who develop anaphylaxis, angioedema, or acute respiratory distress syndrome. (See WARNINGS.)

DESCRIPTION

HERCEPTIN (Trastuzumab) is a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity to a cell-based assay (Kd=5 nM) to the extracellular domain of the human epidermal growth factor receptor 2 protein, HER2.^{1,2} The antibody is an IgG₁ kappa that contains human framework regions with the complementarity-determining regions of a murine antibody (405) that binds to HER2.

The humanized antibody against HER2 is produced by a mammalian cell (Chinese Hamster Ovary [CHO]) suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product.

HERCEPTIN is a sterile, white to pale yellow, preservative-free lyophilized powder for intravenous (IV) administration. The nominal content of each HERCEPTIN vial is 440 mg Trastuzumab, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 400 mg α, α-trehalose dihydrate, and 1.8 mg polysorbate 20, USP. Reconstitution with 20 mL of the supplied Bacteriostatic Water for Injections (BWFI), USP, containing 1.1% benzyl alcohol as a preservative, yields a multi-dose solution containing 21 mg/mL Trastuzumab, at a pH of approximately 6.

CLINICAL PHARMACOLOGY

General

The HER2 (or c-erbB2) proto-oncogene encodes a transmembrane receptor protein of 185 kDa, which is structurally related to the epidermal growth factor receptor. HER2 protein overexpression is observed in 25%-30% of primary breast cancers. HER2 protein overexpression can be determined using immunohistochemistry (IHC) and gene amplification can be determined using fluorescence in situ hybridization (FISH) of fixed tumor blocks.¹ In referenced studies where HERCEPTIN use was not studied,² approximately 96-98% of biopsy specimens that were found to have protein overexpression also had gene amplification and 100% of those with gene amplification also had protein overexpression.^{3,4} The precision of the determination of protein overexpression or gene amplification, however, may vary depending on the sensitivity and specificity of the particular assay and assay procedures used (see PRECAUTIONS). When compared to the referenced studies noted above, the correlation between detectable protein overexpression using immunohistochemistry (IHC) and detectable gene amplification using fluorescence in situ hybridization (FISH) was not as high in the studies of HERCEPTIN clinical trial specimens (see CLINICAL STUDIES: HER2 Detection and HER2 Assay Concordance Studies and PRECAUTIONS: HER2 Testing).

Trastuzumab has been shown, in both *in vitro* assays and in animals, to inhibit the proliferation of human tumor cells that overexpress HER2.⁵

Trastuzumab is a mediator of antibody-dependent cellular cytotoxicity (ADCC).^{6,7} *In vitro*, HERCEPTIN-mediated ADCC has been shown to be preferentially exerted on HER2 overexpressing cancer cells compared with cancer cells that do not overexpress HER2.

Pharmacokinetics

The pharmacokinetics of Trastuzumab were studied in breast cancer patients with metastatic disease. Short duration intravenous infusions of 10 to 500 mg once weekly demonstrated dose-dependent pharmacokinetics. Mean half-life increased and clearance decreased with increasing dose level. The half-life averaged 1.7 and 12 days at the 10 and 500 mg dose levels, respectively. Trastuzumab's volume of distribution was approximately that of serum volume (44 mL/kg). At the highest weekly dose studied (500 mg), mean peak serum concentrations were 377 microgram/mL.

In studies using a loading dose of 4 mg/kg followed by a weekly maintenance dose of 2 mg/kg, a mean half-life of 5.8 days (range=1 to 32 days) was observed. Between Weeks 16 and 32, Trastuzumab serum concentrations reached a steady state with mean trough and peak concentrations of approximately 79 microgram/mL and 123 microgram/mL, respectively.

Detectable concentrations of the circulating extracellular domain of the HER2 receptor (shed antigen) are found in the sera of some patients with HER2 overexpressing tumors. Determination of shed antigen in baseline serum samples revealed that 64% (286/447) of patients had detectable shed antigen, which ranged as high as 1880 ng/mL (median=11 ng/mL). Patients with higher baseline shed antigen levels were more likely to have lower serum trough concentrations. However, with weekly dosing, most patients with elevated shed antigen levels achieved target serum concentrations of Trastuzumab by Week 6.

Data suggest that the disposition of Trastuzumab is not altered based on age or serum creatinine (up to 2.0 mg/dL). No formal interaction studies have been performed.

Mean serum trough concentrations of Trastuzumab, when administered in combination with paclitaxel, were consistently elevated approximately 1.5-fold as compared with serum concentrations of Trastuzumab used in combination with anthracycline plus cyclophosphamide. In primate studies, administration of Trastuzumab with paclitaxel resulted in a reduction in Trastuzumab clearance. Serum levels of Trastuzumab in combination with cisplatin, doxorubicin or epirubicin plus cyclophosphamide did not suggest any interactions; no formal drug interaction studies were performed.

CLINICAL STUDIES

The safety and efficacy of HERCEPTIN were studied in a randomized, controlled clinical trial in combination with chemotherapy (469 patients) and an open-label single agent clinical trial (222 patients). Both trials studied patients with metastatic breast cancer whose tumors overexpress the

HERCEPTIN® (Trastuzumab)

HER2 protein. Patients were eligible if they had 2+ or 3+ levels of overexpression (based on a 0 to 3+ scale) by immunohistochemical assessment of tumor tissue performed by a central testing lab.

A multicenter, randomized, controlled clinical trial was conducted in 469 patients with metastatic breast cancer who had not been previously treated with chemotherapy for metastatic disease.¹ Patients were randomized to receive chemotherapy alone or in combination with HERCEPTIN given intravenously as a 4 mg/kg loading dose followed by weekly doses of HERCEPTIN at 2 mg/kg. For those who had received prior anthracycline therapy in the adjuvant setting, chemotherapy consisted of paclitaxel (175 mg/m² over 3 hours every 21 days for at least six cycles); for all other patients, chemotherapy consisted of anthracycline plus cyclophosphamide (AC: doxorubicin 60 mg/m² or epirubicin 75 mg/m² plus 600 mg/m² cyclophosphamide every 21 days for six cycles). Compared with patients in the AC subgroups (n=281), patients in the paclitaxel subgroup (n=188) were more likely to have had the following: poor prognostic factors (premenopausal status, estrogen or progesterone receptor negative tumors, positive lymph nodes), prior therapy (adjuvant chemotherapy, myeloablative chemotherapy, radiotherapy), and a shorter disease-free interval. Sixty-five percent of patients randomized to receive chemotherapy alone in this study received HERCEPTIN at the time of disease progression as part of a separate extension study.

Compared with patients randomized to chemotherapy alone, the patients randomized to HERCEPTIN and chemotherapy experienced a significantly longer median time to disease progression, a higher overall response rate (ORR), a longer median duration of response, and a longer median survival (see Table 1). These treatment effects were observed both in patients who received HERCEPTIN plus paclitaxel and in those who received HERCEPTIN plus AC, however the magnitude of the effects was greater in the paclitaxel subgroup (see CLINICAL STUDIES: HER2 Detection).

Table 1
Phase III Clinical Efficacy in First-Line Treatment

	Combined Results HERCEPTIN + All Chemo- therapy (n = 235)		Paclitaxel Subgroup HERCEPTIN + Paclitaxel (n = 92)		AC Subgroup HERCEPTIN + AC* (n = 143)	
	All Chemo- therapy (n = 235)	All Chemo- therapy (n = 234)	Paclitaxel (n = 92)	Paclitaxel (n = 96)	AC* (n = 143)	AC (n = 138)
Primary Endpoint						
Time to Progression^{a,c}						
Median (months)	7.2	4.5	6.7	2.5	7.6	5.7
95% confidence interval	6.9, 8.2	4.3, 4.9	5.2, 9.9	2.0, 4.3	7.2, 9.1	4.6, 7.1
p-value (log rank)		<0.0001		<0.0001		0.002
Secondary Endpoints						
Overall Response Rate^b						
Rate (percent)	45	29	38	15	50	38
95% confidence interval	39, 51	23, 35	28, 48	8, 22	42, 58	30, 46
p-value (χ ² -test)		<0.001		<0.001		0.10
Duration of Response^{a,c}						
Median (months)	8.3	5.8	8.3	4.3	8.4	6.4
25%, 75% quartile	5.5, 14.8	3.9, 8.5	5.1, 11.0	3.7, 7.4	5.8, 14.8	4.5, 8.5
Survival Time^c						
Median Survival (months)	25.1	20.3	22.1	18.4	26.8	21.4
95% confidence interval	22.2, 29.5	16.8, 24.2	16.9, 28.6	12.7, 24.4	23.3, 32.9	18.3, 26.6
p-value (log rank)		0.05		0.17		0.16

*AC = anthracycline (doxorubicin or epirubicin) and cyclophosphamide.

^aAssessed by an Independent Response Evaluation Committee.

^cKaplan-Meier Estimate.

HERCEPTIN was studied as a single agent in a multicenter, open-label, single-arm clinical trial in patients with HER2 overexpressing metastatic breast cancer who had relapsed following one or two prior chemotherapy regimens for metastatic disease. Of 222 patients enrolled, 66% had received prior adjuvant chemotherapy, 68% had received two prior chemotherapy regimens for metastatic disease, and 25% had received prior myeloablative treatment with hematopoietic rescue. Patients were treated with a loading dose of 4 mg/kg IV followed by weekly doses of HERCEPTIN at 2 mg/kg IV. The ORR (complete response + partial response), as determined by an Independent Response Evaluation Committee, was 14%, with a 2% complete response rate and a 12% partial response rate. Complete responses were observed only in patients with disease limited to skin and lymph nodes (see CLINICAL STUDIES: HER2 Detection).

HER2 Detection

(See PRECAUTIONS: HER2 Testing)

Detection of HER2 protein overexpression is necessary for selection of patients appropriate for HERCEPTIN therapy (see INDICATIONS). Overexpression of HER2 by tumors was an entry criterion of the two clinical studies described above. In those studies, a research-use-only IHC assay (referred to as the Clinical Trial Assay, CTA) was used.

The commercial assays described below, HercepTest[®] (IHC assay) and PathVysion[®] (FISH assay), are appropriate assays to aid in the selection of patients for HERCEPTIN therapy (see CLINICAL STUDIES: HER2 Detection: HER2 Protein Overexpression Detection Methods and HER2 Gene Amplification Detection Methods). The comparability of either assay with regard to the ability to predict clinical benefit from HERCEPTIN therapy has not been prospectively studied. In addition, the utility of either assay in patients whose tumors would score as 0 or 1+ by the Clinical Trial Assay (CTA) has not been established because patients with tumors that scored as 0 or 1+ were excluded from the clinical studies described.

HER2 Protein Overexpression Detection Methods

HER2 protein overexpression can be established by measuring expressed HER2 protein using IHC methodology. In the clinical trial studies described above, specimens were tested with the CTA and scored as 0, 1+, 2+, or 3+ with 3+ indicating the strongest positivity. Only patients with 2+ or 3+ positive tumors were eligible (about 33% of those screened). Data from the randomized trial suggest that the beneficial treatment effects were largely limited to patients with the highest level of HER2 protein overexpression (3+) (see Table 2). In an exploratory analysis, the relative risk (rr) for time to progression was lower in the patients whose tumors tested as CTA 3+ (rr = 0.42 with 95% CI: 0.33, 0.54) than in those tested as CTA 2+ (rr = 0.76 with 95% CI: 0.50, 1.15). The relative risk represents the risk of progression in the HERCEPTIN plus chemotherapy arm versus the chemotherapy arm. Therefore, a lower ratio represents longer time to progression in the HERCEPTIN arm. In the single-arm study of HERCEPTIN as a single agent, the overall response rate in patients whose tumors tested as CTA 3+ was 18% while in those that tested as CTA 2+, it was 6%.

HercepTest[®], another IHC assay, was assessed for concordance with the CTA (see HER2 Testing: Concordance Studies), but has not been used to assess tumor specimens from the HERCEPTIN clinical studies described above.

HERCEPTIN® (Trastuzumab)

HER2 Gene Amplification Detection Methods

As a surrogate for protein overexpression, measurement of the number of HER2 gene copies using FISH to detect gene amplification may be employed. An exploratory, retrospective assessment of known CTA 2+ or 3+ tumor specimens was performed to detect HER2 gene amplification using PathVision™, a FISH assay. Data from this retrospective analysis involving 660 of 691 (96%) patients enrolled in the clinical studies (all scoring 2+ or 3+ by the CTA) suggested that the beneficial treatment effects were greater in patients whose tumors tested as FISH (+) than in those that were FISH (-); however, time to progression was prolonged for patients on the HERCEPTIN arm, regardless of the FISH result (see Table 2). In the single arm study of HERCEPTIN as a single agent, the overall response rate in patients whose tumors tested as FISH (+) was 20%, while in those tested as FISH (-), there were no responses.

These data are not sufficient to conclude whether FISH testing can distinguish a subpopulation of CTA 2+ patients who would be unlikely to benefit from HERCEPTIN therapy. In addition, there are no data correlating clinical outcome with FISH test results for patients with tumors that scored as 0 or 1+ by CTA; therefore, conclusions regarding the usefulness of FISH in the general population cannot be made.

Table 2
Treatment Effect versus Level of HER2 Expression
Phase III Randomized Trial (N = 469):
HERCEPTIN Plus Chemotherapy versus Chemotherapy

HER2 Assay Result	Number of Patients (N)	Relative Risk** for Time to Disease Progression (95% CI)	Relative Risk** for Mortality (95% CI)
CTA 2+ or 3+ FISH (+)	469	0.49 (0.40, 0.61)	0.80 (0.64, 1.00)
FISH (-)	325	0.44 (0.34, 0.57)	0.70 (0.53, 0.91)
FISH (+)	126	0.62 (0.42, 0.94)	1.06 (0.70, 1.63)
CTA 2+ FISH (+)	120	0.76 (0.50, 1.15)	1.26 (0.82, 1.94)
FISH (-)	32	0.54 (0.21, 1.35)	1.31 (0.53, 3.27)
FISH (+)	83	0.77 (0.48, 1.25)	1.11 (0.68, 1.82)
CTA 3+ FISH (+)	349	0.42 (0.33, 0.54)	0.70 (0.51, 0.90)
FISH (-)	293	0.42 (0.32, 0.55)	0.67 (0.51, 0.89)
FISH (+)	43	0.43 (0.20, 0.94)	0.88 (0.39, 1.98)

*FISH testing results were available for 451 of the 469 patients enrolled on study.

**The relative risk represents the risk of progression or death in the HERCEPTIN plus chemotherapy arm versus the chemotherapy arm.

HER2 Assay Concordance Studies (See PRECAUTIONS: HER2 Testing)

Immunohistochemistry: The DAKO HercepTest™, an IHC test for detecting HER2 protein overexpression, has not been directly studied for its ability to predict HERCEPTIN treatment effect, but has been compared to the CTA on over 500 breast cancer histology specimens obtained from the National Cancer Institute Cooperative Breast Cancer Tissue Resource. Based upon these results, of specimens testing 3+ (strongly positive) on the HercepTest™, 82% were 3+ (i.e., the reading most associated with clinical benefit), 12% were 2+, and 6% were 0 or 1+ on the CTA. The 6% of HercepTest™ 3+ specimens that were CTA 0 or 1+ would be expected to represent 2% of the 0 and 1+ population. Of specimens testing 2+ (weakly positive) on the HercepTest™, 14% were 3+, 20% were 2+, and 66% were 0 or 1+ on the CTA. Of specimens testing 0 or 1+ on the HercepTest™, 2% were 2+, 6% were 2+, and 92% were 0 or 1+ on the CTA.

Fluorescence In Situ Hybridization: The Vysis PathVision™ HER2 DNA Probe, a FISH test for detecting HER2 gene amplification, was compared with the CTA on over 500 breast cancer histology specimens originally submitted for potential enrollment in the HERCEPTIN trials. A HER2:CEP17 ratio of ≥2 was defined as FISH positive (+). Based on these results, of specimens testing FISH (+) by PathVision™, 81% were 3+, 10% were 2+, and 9% were 0 or 1+ on the CTA. The 9% of FISH (+) specimens that were CTA 0 or 1+ would be expected to represent 3% of the total CTA 0 or 1+ population. Of specimens testing FISH (-) by PathVision™, 3% were 3+, 10% were 2+, and 87% were 0 or 1+ on the CTA.

INDICATIONS AND USAGE

HERCEPTIN as a single agent is indicated for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have received one or more chemotherapy regimens for their metastatic disease. HERCEPTIN in combination with paclitaxel is indicated for treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have not received chemotherapy for their metastatic disease. HERCEPTIN should be used in patients whose tumors have been evaluated with an assay validated to predict HER2 protein overexpression (see PRECAUTIONS: HER2 Testing and CLINICAL STUDIES: HER2 Detection).

CONTRAINDICATIONS

None known.

WARNINGS

Cardiotoxicity:

Signs and symptoms of cardiac dysfunction, such as dyspnea, increased cough, paroxysmal nocturnal dyspnea, peripheral edema, S₃ gallop, or reduced ejection fraction, have been observed in patients treated with HERCEPTIN. Congestive heart failure associated with HERCEPTIN therapy may be severe and has been associated with disabling cardiac failure, death, and mural thrombosis leading to stroke (see BOXED WARNINGS: CARDIOMYOPATHY). The clinical status of patients in the trials who developed congestive heart failure was classified for severity using the New York Heart Association classification system (I-IV, where IV is the most severe level of cardiac failure). (See Table 3.)

Table 3
Incidence and Severity of Cardiac Dysfunction

	HERCEPTIN ^a alone n=213	HERCEPTIN + Paclitaxel ^b n=91	Paclitaxel ^b n=95	HERCEPTIN + Anthracycline + cyclophosphamide ^b n=143	Anthracycline + cyclophosphamide ^b n=135
Any Cardiac Dysfunction	7%	11%	1%	28%	7%
Class III-IV	5%	4%	1%	19%	3%

^aOpen-label, single-agent Phase II study (94% received prior anthracyclines).

^bRandomized Phase III study comparing chemotherapy plus HERCEPTIN to chemotherapy alone, where chemotherapy is either anthracycline/cyclophosphamide or paclitaxel.

Candidates for treatment with HERCEPTIN should undergo thorough baseline cardiac assessment including history and physical exam and one or more of the following: EKG, echocardiogram, and MUGA scan. There are no data regarding the most appropriate method of evaluation for the identification of patients at risk for developing cardiotoxicity. Monitoring may not identify all patients who will develop cardiac dysfunction.

HERCEPTIN® (Trastuzumab)

Extreme caution should be exercised in treating patients with pre-existing cardiac dysfunction.

Patients receiving HERCEPTIN should undergo frequent monitoring for deteriorating cardiac function.

The probability of cardiac dysfunction was highest in patients who received HERCEPTIN concurrently with anthracyclines. The data suggest that advanced age may increase the probability of cardiac dysfunction.

Pre-existing cardiac disease or prior cardiotoxic therapy (e.g., anthracycline or radiation therapy to the chest) may decrease the ability to tolerate HERCEPTIN therapy; however, the data are not adequate to evaluate the correlation between HERCEPTIN-induced cardiotoxicity and these factors.

Discontinuation of HERCEPTIN therapy should be strongly considered in patients who develop clinically significant congestive heart failure. In the clinical trials, most patients with cardiac dysfunction responded to appropriate medical therapy often including discontinuation of HERCEPTIN. The safety of continuation or resumption of HERCEPTIN in patients who have previously experienced cardiac toxicity has not been studied. There are insufficient data regarding discontinuation of HERCEPTIN therapy in patients with asymptomatic decreases in ejection fraction; such patients should be closely monitored for evidence of clinical deterioration.

Hypersensitivity Reactions Including Anaphylaxis:

Severe hypersensitivity reactions have been infrequently reported in patients treated with HERCEPTIN (see BOXED WARNINGS: HYPERSENSITIVITY REACTIONS INCLUDING ANAPHYLAXIS). Signs and symptoms include anaphylaxis, urticaria, bronchospasm, angioedema, and/or hypotension. In some cases, the reactions have been fatal. The onset of symptoms generally occurred during an infusion, but there have also been reports of symptom onset after the completion of an infusion. Reactions were most commonly reported in association with the initial infusion.

HERCEPTIN infusion should be interrupted in all patients with severe hypersensitivity reactions. In the event of a hypersensitivity reaction, appropriate medical therapy should be administered, which may include epinephrine, corticosteroids, diphenhydramine, bronchodilators, and oxygen. Patients should be evaluated and carefully monitored until complete resolution of signs and symptoms.

There are no data regarding the most appropriate method of identification of patients who may safely be retreated with HERCEPTIN after experiencing a severe hypersensitivity reaction. HERCEPTIN has been readministered to some patients who fully recovered from a previous severe reaction. Prior to readministration of HERCEPTIN, the majority of these patients were prophylactically treated with pre-medications including antihistamines and/or corticosteroids. While some of these patients tolerated retreatment, others had severe reactions again despite the use of prophylactic pre-medications.

Infusion Reactions:

In the postmarketing setting, rare occurrences of severe infusion reactions leading to a fatal outcome have been associated with the use of HERCEPTIN. (See BOXED WARNINGS: INFUSION REACTIONS.)

In clinical trials, infusion reactions consisted of a symptom complex characterized by fever and chills, and on occasion included nausea, vomiting, pain (in some cases at tumor sites), headache, dizziness, dyspnea, hypotension, rash, and asthenia. These reactions were usually mild to moderate in severity. (See ADVERSE REACTIONS.)

However, in postmarketing reports, more severe adverse reactions to HERCEPTIN infusion were observed and included bronchospasm, hypoxia, and severe hypotension. These severe reactions were usually associated with the initial infusion of HERCEPTIN and generally occurred during or immediately following the infusion. However, the onset and clinical course were variable. For some patients, symptoms progressively worsened and led to further pulmonary complications. (See WARNINGS: Pulmonary Events.) In other patients with acute onset of signs and symptoms, initial improvement was followed by clinical deterioration. Delayed post-infusion events with rapid clinical deterioration have also been reported. Rarely, severe infusion reactions culminated in death within hours or up to one week following an infusion.

Some severe reactions have been treated successfully with interruption of the HERCEPTIN infusion and supportive therapy including oxygen, intravenous fluids, beta-agonists, and corticosteroids.

There are no data regarding the most appropriate method of identification of patients who may safely be retreated with HERCEPTIN after experiencing a severe infusion reaction. HERCEPTIN has been readministered to some patients who fully recovered from the previous severe reaction. Prior to readministration of HERCEPTIN, the majority of these patients were prophylactically treated with pre-medications including antihistamines and/or corticosteroids. While some of these patients tolerated retreatment, others had severe reactions again despite the use of prophylactic pre-medications.

Pulmonary Events:

Severe pulmonary events leading to death have been reported rarely with the use of HERCEPTIN in the postmarketing setting. Signs, symptoms and clinical findings include dyspnea, pulmonary infiltrates, pleural effusions, non-cardiogenic pulmonary edema, pulmonary insufficiency and hypoxia, and acute respiratory distress syndrome. These events may or may not occur as sequelae of infusion reactions. (See WARNINGS: Infusion Reactions.) Patients with symptomatic intrinsic lung disease or with extensive tumor involvement of the lungs, resulting in dyspnea at rest, may be at greater risk of severe reactions.

Other severe events reported rarely in the postmarketing setting include pneumonitis and pulmonary fibrosis.

PRECAUTIONS

General:

HERCEPTIN therapy should be used with caution in patients with known hypersensitivity to Trastuzumab, Chinese Hamster Ovary cell proteins, or any component of this product.

HER2 Testing:

Assessment for HER2 overexpression should be performed by laboratories with demonstrated proficiency in the specific technology being utilized. Improper assay performance, including use of suboptimally fixed tissue, failure to utilize specified reagents, deviation from specific assay instructions, and failure to include appropriate controls for assay validation, can lead to unreliable results. Refer to the HercepTest™ and PathVision™ package inserts for full instructions on assay performance (see CLINICAL STUDIES: HER2 Detection).

Drug Interactions:

There have been no formal drug interaction studies performed with HERCEPTIN in humans. Administration of paclitaxel in combination with HERCEPTIN resulted in a two-fold decrease in HERCEPTIN clearance in a non-human primate study and in a 1.5-fold increase in HERCEPTIN serum levels in clinical studies. (See PHARMACOKINETICS.)

Benzyl Alcohol:

For patients with a known hypersensitivity to benzyl alcohol (the preservative in Bacteriostatic Water for Injection) reconstitute HERCEPTIN with Sterile Water for Injection (SWFI), USP. DISCARD THE SWFI-RECONSTITUTED HERCEPTIN VIAL FOLLOWING A SINGLE USE.

Carcinogenesis, Mutagenesis, Impairment of Fertility:

Carcinogenesis:

HERCEPTIN has not been tested for its carcinogenic potential.

Mutagenesis:

No evidence of mutagenic activity was observed in Ames tests using six different test strains of bacteria, with and without metabolic activation, at concentrations of up to 5000 µg/mL Trastuzumab. Human peripheral blood lymphocytes treated *in vitro* at concentrations of up to 5000 µg/plate

HERCEPTIN® (Trastuzumab)

HER2 Gene Amplification Detection Methods

As a surrogate for protein overexpression, measurement of the number of HER2 gene copies using FISH to detect gene amplification may be employed. An exploratory, retrospective assessment of known CTA 2+ or 3+ tumor specimens was performed to detect HER2 gene amplification using PathVision™, a FISH assay. Data from this retrospective analysis involving 660 of 691 (96%) patients enrolled in the clinical studies (all scoring 2+ or 3+ by the CTA) suggested that the beneficial treatment effects were greater in patients whose tumors tested as FISH (+) than in those that were FISH (-); however, time to progression was prolonged for patients on the HERCEPTIN arm, regardless of the FISH result (see Table 2). In the single arm study of HERCEPTIN as a single agent, the overall response rate in patients whose tumors tested as FISH (+) was 20%, while in those tested as FISH (-), there were no responses.

These data are not sufficient to conclude whether FISH testing can distinguish a subpopulation of CTA 2+ patients who would be unlikely to benefit from HERCEPTIN therapy. In addition, there are no data correlating clinical outcome with FISH test results for patients with tumors that scored as 0 or 1+ by CTA; therefore, conclusions regarding the usefulness of FISH in the general population cannot be made.

Table 2
Treatment Effect versus Level of HER2 Expression
Phase III Randomized Trial (N = 469):
HERCEPTIN Plus Chemotherapy versus Chemotherapy

HER2 Assay Result	Number of Patients (N)	Relative Risk** for Time to Disease Progression (95% CI)	Relative Risk** for Mortality (95% CI)
CTA 2+ or 3+ FISH (+)* FISH (-)*	469 325 126	0.49 (0.40, 0.61) 0.44 (0.34, 0.57) 0.62 (0.42, 0.94)	0.80 (0.64, 1.00) 0.70 (0.53, 0.91) 1.06 (0.70, 1.63)
CTA 2+ FISH (+) FISH (-)	120 32 88	0.76 (0.50, 1.15) 0.54 (0.21, 1.35) 0.77 (0.48, 1.25)	1.26 (0.82, 1.94) 1.31 (0.53, 3.27) 1.11 (0.68, 1.82)
CTA 3+ FISH (+) FISH (-)	349 293 43	0.42 (0.33, 0.54) 0.42 (0.32, 0.55) 0.43 (0.20, 0.94)	0.70 (0.51, 0.90) 0.67 (0.51, 0.89) 0.88 (0.39, 1.98)

*FISH testing results were available for 451 of the 469 patients enrolled on study.

**The relative risk represents the risk of progression or death in the HERCEPTIN plus chemotherapy arm versus the chemotherapy arm.

HER2 Assay Concordance Studies (See PRECAUTIONS: HER2 Testing)

Immunohistochemistry: The DAKO HercepTest™, an IHC test for detecting HER2 protein overexpression, has not been directly studied for its ability to predict HERCEPTIN treatment effect, but has been compared to the CTA on over 500 breast cancer histology specimens obtained from the National Cancer Institute Cooperative Breast Cancer Tissue Resource. Based upon these results, of specimens testing 3+ (strongly positive) on the HercepTest™, 82% were 3+ (i.e., the reading most associated with clinical benefit), 12% were 2+, and 6% were 0 or 1+ on the CTA. The 6% of HercepTest™ 3+ specimens that were CTA 0 or 1+ would be expected to represent 2% of the 0 and 1+ population. Of specimens testing 2+ (weakly positive) on the HercepTest™, 14% were 3+, 20% were 2+, and 66% were 0 or 1+ on the CTA. Of specimens testing 0 or 1+ on the HercepTest™, 2% were 3+, 6% were 2+, and 92% were 0 or 1+ on the CTA.

Fluorescence in situ Hybridization: The Vysis PathVision™ HER2 DNA Probe, a FISH test for detecting HER2 gene amplification, was compared with the CTA on over 500 breast cancer histology specimens originally submitted for potential enrollment in the HERCEPTIN trials. A HER2:CEP17 ratio of ≥2 was defined as FISH positive (+). Based on these results, of specimens testing FISH (+) by PathVision™, 81% were 3+, 10% were 2+, and 9% were 0 or 1+ on the CTA. The 9% of FISH (+) specimens that were CTA 0 or 1+ would be expected to represent 3% of the total CTA 0 or 1+ population. Of specimens testing FISH (-) by PathVision™, 3% were 3+, 10% were 2+, and 87% were 0 or 1+ on the CTA.

INDICATIONS AND USAGE

HERCEPTIN as a single agent is indicated for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have received one or more chemotherapy regimens for their metastatic disease. HERCEPTIN in combination with paclitaxel is indicated for treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have not received chemotherapy for their metastatic disease. HERCEPTIN should be used in patients whose tumors have been evaluated with an assay validated to predict HER2 protein overexpression (see PRECAUTIONS: HER2 Testing and CLINICAL STUDIES: HER2 Detection).

CONTRAINDICATIONS

None known.

WARNINGS

Cardiotoxicity:

Signs and symptoms of cardiac dysfunction, such as dyspnea, increased cough, paroxysmal nocturnal dyspnea, peripheral edema, S₃ gallop, or reduced ejection fraction, have been observed in patients treated with HERCEPTIN. Congestive heart failure associated with HERCEPTIN therapy may be severe and has been associated with disabling cardiac failure, death, and mural thrombosis leading to stroke (see BOXED WARNINGS: CARDIOMYOPATHY). The clinical status of patients in the trials who developed congestive heart failure was classified for severity using the New York Heart Association classification system (I-IV, where IV is the most severe level of cardiac failure). (See Table 3.)

Table 3
Incidence and Severity of Cardiac Dysfunction

	HERCEPTIN ^a alone n=213	HERCEPTIN + Paclitaxel ^b n=91	Paclitaxel ^b n=95	HERCEPTIN + Anthracycline + cyclophosphamide ^b n=143	Anthracycline + cyclophosphamide ^b n=135
Any Cardiac Dysfunction	7%	11%	1%	28%	7%
Class III-IV	5%	4%	1%	19%	3%

^aOpen-label, single-agent Phase II study (94% received prior anthracyclines).

^bRandomized Phase III study comparing chemotherapy plus HERCEPTIN to chemotherapy alone, where chemotherapy is either anthracycline/cyclophosphamide or paclitaxel.

Candidates for treatment with HERCEPTIN should undergo thorough baseline cardiac assessment including history and physical exam and one or more of the following: EKG, echocardiogram, and MUGA scan. There are no data regarding the most appropriate method of evaluation for the identification of patients at risk for developing cardiotoxicity. Monitoring may not identify all patients who will develop cardiac dysfunction.

HERCEPTIN® (Trastuzumab)

Extreme caution should be exercised in treating patients with pre-existing cardiac dysfunction.

Patients receiving HERCEPTIN should undergo frequent monitoring for deteriorating cardiac function.

The probability of cardiac dysfunction was highest in patients who received HERCEPTIN concurrently with anthracyclines. The data suggest that advanced age may increase the probability of cardiac dysfunction.

Pre-existing cardiac disease or prior cardiotoxic therapy (e.g., anthracycline or radiation therapy to the chest) may decrease the ability to tolerate HERCEPTIN therapy; however, the data are not adequate to evaluate the correlation between HERCEPTIN-induced cardiotoxicity and these factors.

Discontinuation of HERCEPTIN therapy should be strongly considered in patients who develop clinically significant congestive heart failure. In the clinical trials, most patients with cardiac dysfunction responded to appropriate medical therapy often including discontinuation of HERCEPTIN. The safety of continuation or resumption of HERCEPTIN in patients who have previously experienced cardiac toxicity has not been studied. There are insufficient data regarding discontinuation of HERCEPTIN therapy in patients with asymptomatic decreases in ejection fraction; such patients should be closely monitored for evidence of clinical deterioration.

Hypersensitivity Reactions Including Anaphylaxis:

Severe hypersensitivity reactions have been infrequently reported in patients treated with HERCEPTIN (see BOXED WARNINGS: HYPERSENSITIVITY REACTIONS INCLUDING ANAPHYLAXIS). Signs and symptoms include anaphylaxis, urticaria, bronchospasm, angioedema, and/or hypotension. In some cases, the reactions have been fatal. The onset of symptoms generally occurred during an infusion, but there have also been reports of symptom onset after the completion of an infusion. Reactions were most commonly reported in association with the initial infusion.

HERCEPTIN infusion should be interrupted in all patients with severe hypersensitivity reactions. In the event of a hypersensitivity reaction, appropriate medical therapy should be administered, which may include epinephrine, corticosteroids, diphenhydramine, bronchodilators, and oxygen. Patients should be evaluated and carefully monitored until complete resolution of signs and symptoms.

There are no data regarding the most appropriate method of identification of patients who may safely be retreated with HERCEPTIN after experiencing a severe hypersensitivity reaction. HERCEPTIN has been readministered to some patients who fully recovered from a previous severe reaction. Prior to readministration of HERCEPTIN, the majority of these patients were prophylactically treated with pre-medications including antihistamines and/or corticosteroids. While some of these patients tolerated retreatment, others had severe reactions again despite the use of prophylactic pre-medications.

Infusion Reactions:

In the postmarketing setting, rare occurrences of severe infusion reactions leading to a fatal outcome have been associated with the use of HERCEPTIN. (See BOXED WARNINGS: INFUSION REACTIONS.)

In clinical trials, infusion reactions consisted of a symptom complex characterized by fever and chills, and on occasion included nausea, vomiting, pain (in some cases at tumor sites), headache, dizziness, dyspnea, hypotension, rash, and asthenia. These reactions were usually mild to moderate in severity. (See ADVERSE REACTIONS.)

However, in postmarketing reports, more severe adverse reactions to HERCEPTIN infusion were observed and included bronchospasm, hypoxia, and severe hypotension. These severe reactions were usually associated with the initial infusion of HERCEPTIN and generally occurred during or immediately following the infusion. However, the onset and clinical course were variable. For some patients, symptoms progressively worsened and led to further pulmonary complications. (See WARNINGS: Pulmonary Events.) In other patients with acute onset of signs and symptoms, initial improvement was followed by clinical deterioration. Delayed post-infusion events with rapid clinical deterioration have also been reported. Rarely, severe infusion reactions culminated in death within hours or up to one week following an infusion.

Some severe reactions have been treated successfully with interruption of the HERCEPTIN infusion and supportive therapy including oxygen, intravenous fluids, beta-agonists, and corticosteroids.

There are no data regarding the most appropriate method of identification of patients who may safely be retreated with HERCEPTIN after experiencing a severe infusion reaction. HERCEPTIN has been readministered to some patients who fully recovered from the previous severe reaction. Prior to readministration of HERCEPTIN, the majority of these patients were prophylactically treated with pre-medications including antihistamines and/or corticosteroids. While some of these patients tolerated retreatment, others had severe reactions again despite the use of prophylactic pre-medications.

Pulmonary Events:

Severe pulmonary events leading to death have been reported rarely with the use of HERCEPTIN in the postmarketing setting. Signs, symptoms and clinical findings include dyspnea, pulmonary infiltrates, pleural effusions, non-cardiogenic pulmonary edema, pulmonary insufficiency and hypoxia, and acute respiratory distress syndrome. These events may or may not occur as sequelae of infusion reactions. (See WARNINGS: Infusion Reactions.) Patients with symptomatic intrinsic lung disease or with extensive tumor involvement of the lungs, resulting in dyspnea at rest, may be at greater risk of severe reactions.

Other severe events reported rarely in the postmarketing setting include pneumonitis and pulmonary fibrosis.

PRECAUTIONS

General:

HERCEPTIN therapy should be used with caution in patients with known hypersensitivity to Trastuzumab, Chinese Hamster Ovary cell proteins, or any component of this product.

HER2 Testing:

Assessment for HER2 overexpression should be performed by laboratories with demonstrated proficiency in the specific technology being utilized. Improper assay performance, including use of suboptimally fixed tissue, failure to utilize specified reagents, deviation from specific assay instructions, and failure to include appropriate controls for assay validation, can lead to unreliable results. Refer to the HercepTest™ and PathVision™ package inserts for full instructions on assay performance (see CLINICAL STUDIES: HER2 Detection).

Drug Interactions:

There have been no formal drug interaction studies performed with HERCEPTIN in humans. Administration of paclitaxel in combination with HERCEPTIN resulted in a two-fold decrease in HERCEPTIN clearance in a non-human primate study and in a 1.5-fold increase in HERCEPTIN serum levels in clinical studies. (See PHARMACOKINETICS.)

Benzyl Alcohol:

For patients with a known hypersensitivity to benzyl alcohol (the preservative in Bacteriostatic Water for Injection) reconstitute HERCEPTIN with Sterile Water for Injection (SWFI), USP. DISCARD THE SWFI-RECONSTITUTED HERCEPTIN VIAL FOLLOWING A SINGLE USE.

Carcinogenesis, Mutagenesis, Impairment of Fertility:

Carcinogenesis:

HERCEPTIN has not been tested for its carcinogenic potential.

Mutagenesis:

No evidence of mutagenic activity was observed in Ames tests using six different test strains of bacteria, with and without metabolic activation, at concentrations of up to 5000 µg/mL Trastuzumab. Human peripheral blood lymphocytes treated *in vitro* at concentrations of up to 5000 µg/plate

HERCEPTIN® (Trastuzumab)

Trastuzumab, with and without metabolic activation, revealed no evidence of mutagenic potential. In an *in vivo* mutagenic assay (the micronucleus assay), no evidence of chromosomal damage to mouse bone marrow cells was observed following bolus intravenous doses of up to 118 mg/kg Trastuzumab.

Impairment of Fertility:

A fertility study has been conducted in female cynomolgus monkeys at doses up to 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN and has revealed no evidence of impaired fertility.

Pregnancy Category B:

Reproduction studies have been conducted in cynomolgus monkeys at doses up to 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN and have revealed no evidence of impaired fertility or harm to the fetus. However, HER2 protein expression is high in many embryonic tissues including cardiac and neural tissues; in mutant mice lacking HER2, embryos died in early gestation.⁹ Placental transfer of HERCEPTIN during the early (Days 20–50 of gestation) and late (Days 120–150 of gestation) fetal development period was observed in monkeys. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

Nursing Mothers:

A study conducted in lactating cynomolgus monkeys at doses 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN demonstrated that Trastuzumab is secreted in the milk. The presence of Trastuzumab in the serum of infant monkeys was not associated with any adverse effects on their growth or development from birth to 3 months of age. It is not known whether HERCEPTIN is excreted in human milk. Because human IgG is excreted in human milk, and the potential for absorption and harm to the infant is unknown, women should be advised to discontinue nursing during HERCEPTIN therapy and for 6 months after the last dose of HERCEPTIN.

Pediatric Use:

The safety and effectiveness of HERCEPTIN in pediatric patients have not been established.

Geriatric Use:

HERCEPTIN has been administered to 133 patients who were 65 years of age or over. The risk of cardiac dysfunction may be increased in geriatric patients. The reported clinical experience is not adequate to determine whether older patients respond differently from younger patients.

ADVERSE REACTIONS

In clinical studies, a total of 958 patients have received HERCEPTIN alone or in combination with chemotherapy. Data in Table 4 are based on the experience with the recommended dosing regimen for HERCEPTIN in the randomized controlled clinical trial in 234 patients who received HERCEPTIN in combination with chemotherapy and four open-label studies of HERCEPTIN as a single agent in 352 patients at doses of 10–500 mg administered weekly.

Cardiac Failure/Dysfunction:

For a description of cardiac toxicities, see **BOXED WARNINGS: CARDIOMYOPATHY** and **WARNINGS: Cardiotoxicity**.

Anemia and Leukopenia:

An increased incidence of anemia and leukopenia was observed in the treatment group receiving HERCEPTIN and chemotherapy, especially in the HERCEPTIN and AC subgroup, compared with the treatment group receiving chemotherapy alone. The majority of these cytopenic events were mild or moderate in intensity, reversible, and none resulted in discontinuation of therapy with HERCEPTIN.

Hematologic toxicity is infrequent following the administration of HERCEPTIN as a single agent, with an incidence of Grade III toxicities for WBC, platelets, hemoglobin all <1%. No Grade IV toxicities were observed.

Diarrhea:

Of patients treated with HERCEPTIN as a single agent, 25% experienced diarrhea. An increased incidence of diarrhea, primarily mild to moderate in severity, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infection:

An increased incidence of infections, primarily mild upper respiratory infections of minor clinical significance or catheter infections, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infusion Reactions:

During the first infusion with HERCEPTIN, a symptom complex most commonly consisting of chills and/or fever was observed in about 40% of patients in clinical trials. The symptoms were usually mild to moderate in severity and were treated with acetaminophen, diphenhydramine, and meperidine (with or without reduction in the rate of HERCEPTIN infusion). HERCEPTIN discontinuation was infrequent. Other signs and/or symptoms may include nausea, vomiting, pain (in some cases at tumor sites), rigors, headache, dizziness, dyspnea, hypotension, rash and asthenia. The symptoms occurred infrequently with subsequent HERCEPTIN infusions. (See **BOXED WARNINGS: INFUSION REACTIONS** and **WARNINGS: Infusion Reactions**.)

Additional adverse reactions have been identified during postmarketing use of HERCEPTIN. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to HERCEPTIN exposure. Decisions to include these reactions in labeling are typically based on one or more of the following factors: (1) seriousness of the reaction, (2) frequency of reporting, or (3) strength of causal connection to HERCEPTIN.

Hypersensitivity Reactions Including Anaphylaxis**Pulmonary Events:**

In the postmarketing setting, severe hypersensitivity reactions (including anaphylaxis), infusion reactions, and pulmonary adverse events have been reported (see **BOXED WARNINGS: HYPERSENSITIVITY REACTIONS INCLUDING ANAPHYLAXIS** and **WARNINGS: Hypersensitivity Reactions Including Anaphylaxis**). These events include anaphylaxis, angioedema, bronchospasm, hypotension, hypoxia, dyspnea, pulmonary infiltrates, pleural effusions, non-cardiogenic pulmonary edema and acute respiratory distress syndrome. For a detailed description, see **WARNINGS**.

Glomerulopathy:

In the postmarketing setting, rare cases of nephrotic syndrome with pathologic evidence of glomerulopathy have been reported. The time to onset ranged from 4 months to approximately 18 months from initiation of HERCEPTIN therapy. Pathologic findings included membranous glomerulonephritis, focal glomerulosclerosis and fibrillary glomerulonephritis. Complications included volume overload and congestive heart failure.

HERCEPTIN® (Trastuzumab)

Table 4
Adverse Events Occurring in ≥5% of Patients or at Increased Incidence in the HERCEPTIN Arm of the Randomized Study (Percent of Patients)

	Single Agent n=352	HERCEPTIN + Paclitaxel n=91	Paclitaxel Alone n=95	HERCEPTIN + AC n=143	AC Alone n=135
Body as a Whole					
Pain	47	61	62	57	42
Asthenia	42	62	57	54	55
Fever	36	49	23	56	34
Chills	32	41	4	35	11
Headache	26	36	28	44	31
Abdominal pain	22	34	22	23	18
Back pain	22	34	30	27	15
Infection	20	47	27	47	31
Flu syndrome	10	12	5	12	6
Accidental injury	6	13	3	9	4
Allergic reaction	3	8	2	4	2
Cardiovascular					
Tachycardia	5	12	4	10	5
Congestive heart failure	7	11	1	28	7
Digestive					
Nausea	33	51	9	76	77
Diarrhea	25	45	29	45	26
Vomiting	23	37	28	53	49
Nausea and vomiting	8	14	11	18	9
Anorexia	14	24	16	31	26
Heme & Lymphatic					
Anemia	4	14	9	36	26
Leukopenia	3	24	17	52	34
Metabolic					
Peripheral edema	10	22	20	20	17
Edema	8	10	8	11	5
Musculoskeletal					
Bone pain	7	24	18	7	7
Arthralgia	6	37	21	8	9
Nervous					
Insomnia	14	25	13	29	15
Dizziness	13	22	24	24	18
Paresthesia	9	48	39	17	11
Depression	6	12	13	20	12
Peripheral neuritis	2	23	16	2	2
Neuropathy	1	13	5	4	4
Respiratory					
Cough increased	26	41	22	43	29
Dyspnea	22	27	26	42	25
Rhinitis	14	22	5	22	16
Pharyngitis	12	22	14	30	18
Sinusitis	9	21	7	13	6
Skin					
Rash	18	38	18	27	17
Herpes simplex	2	12	3	7	9
Acne	2	11	3	3	<1
Urogenital					
Urinary tract infection	5	18	14	13	7

HERCEPTIN® (Trastuzumab)

Trastuzumab, with and without metabolic activation, revealed no evidence of mutagenic potential. In an *in vivo* mutagenic assay (the micronucleus assay), no evidence of chromosomal damage to mouse bone marrow cells was observed following bolus intravenous doses of up to 118 mg/kg Trastuzumab.

Impairment of Fertility:

A fertility study has been conducted in female cynomolgus monkeys at doses up to 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN and has revealed no evidence of impaired fertility.

Pregnancy Category B:

Reproduction studies have been conducted in cynomolgus monkeys at doses up to 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN and have revealed no evidence of impaired fertility or harm to the fetus. However, HER2 protein expression is high in many embryonic tissues including cardiac and neural tissues; in mutant mice lacking HER2, embryos died in early gestation.²² Placental transfer of HERCEPTIN during the early (Days 20–50 of gestation) and late (Days 120–150 of gestation) fetal development period was observed in monkeys. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

Nursing Mothers:

A study conducted in lactating cynomolgus monkeys at doses 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN demonstrated that Trastuzumab is secreted in the milk. The presence of Trastuzumab in the serum of infant monkeys was not associated with any adverse effects on their growth or development from birth to 3 months of age. It is not known whether HERCEPTIN is excreted in human milk. Because human IgG is excreted in human milk, and the potential for absorption and harm to the infant is unknown, women should be advised to discontinue nursing during HERCEPTIN therapy and for 6 months after the last dose of HERCEPTIN.

Pediatric Use:

The safety and effectiveness of HERCEPTIN in pediatric patients have not been established.

Geriatric Use:

HERCEPTIN has been administered to 133 patients who were 65 years of age or over. The risk of cardiac dysfunction may be increased in geriatric patients. The reported clinical experience is not adequate to determine whether older patients respond differently from younger patients.

ADVERSE REACTIONS

In clinical studies, a total of 958 patients have received HERCEPTIN alone or in combination with chemotherapy. Data in Table 4 are based on the experience with the recommended dosing regimen for HERCEPTIN in the randomized controlled clinical trial in 234 patients who received HERCEPTIN in combination with chemotherapy and four open-label studies of HERCEPTIN as a single agent in 352 patients at doses of 10–500 mg administered weekly.

Cardiac Failure/Dysfunction:

For a description of cardiac toxicities, see **BOXED WARNINGS: CARDIOMYOPATHY** and **WARNINGS: Cardiotoxicity**.

Anemia and Leukopenia:

An increased incidence of anemia and leukopenia was observed in the treatment group receiving HERCEPTIN and chemotherapy, especially in the HERCEPTIN and AC subgroup, compared with the treatment group receiving chemotherapy alone. The majority of these cytopenic events were mild or moderate in intensity, reversible, and none resulted in discontinuation of therapy with HERCEPTIN.

Hematologic toxicity is infrequent following the administration of HERCEPTIN as a single agent, with an incidence of Grade III toxicities for WBC, platelets, hemoglobin all <1%. No Grade IV toxicities were observed.

Diarrhea:

Of patients treated with HERCEPTIN as a single agent, 25% experienced diarrhea. An increased incidence of diarrhea, primarily mild to moderate in severity, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infection:

An increased incidence of infections, primarily mild upper respiratory infections of minor clinical significance or catheter infections, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infusion Reactions:

During the first infusion with HERCEPTIN, a symptom complex most commonly consisting of chills and/or fever was observed in about 40% of patients in clinical trials. The symptoms were usually mild to moderate in severity and were treated with acetaminophen, diphenhydramine, and meperidine (with or without reduction in the rate of HERCEPTIN infusion). HERCEPTIN discontinuation was infrequent. Other signs and/or symptoms may include nausea, vomiting, pain (in some cases at tumor sites), rigors, headache, dizziness, dyspnea, hypotension, rash and asthenia. The symptoms occurred infrequently with subsequent HERCEPTIN infusions. (See **BOXED WARNINGS: INFUSION REACTIONS** and **WARNINGS: Infusion Reactions**.)

Additional adverse reactions have been identified during postmarketing use of HERCEPTIN. Because these reactions are reported voluntarily from a population of uncertain size, it is not always possible to reliably estimate their frequency or establish a causal relationship to HERCEPTIN exposure. Decisions to include these reactions in labeling are typically based on one or more of the following factors: (1) seriousness of the reaction, (2) frequency of reporting, or (3) strength of causal connection to HERCEPTIN.

Hypersensitivity Reactions Including Anaphylaxis**Pulmonary Events:**

In the postmarketing setting, severe hypersensitivity reactions (including anaphylaxis), infusion reactions, and pulmonary adverse events have been reported (see **BOXED WARNINGS: HYPERSENSITIVITY REACTIONS INCLUDING ANAPHYLAXIS** and **WARNINGS: Hypersensitivity Reactions Including Anaphylaxis**). These events include anaphylaxis, angioedema, bronchospasm, hypotension, hypoxia, dyspnea, pulmonary infiltrates, pleural effusions, non-cardiogenic pulmonary edema and acute respiratory distress syndrome. For a detailed description, see **WARNINGS**.

Glomerulopathy:

In the postmarketing setting, rare cases of nephrotic syndrome with pathologic evidence of glomerulopathy have been reported. The time to onset ranged from 4 months to approximately 18 months from initiation of HERCEPTIN therapy. Pathologic findings included membranous glomerulonephritis, focal glomerulosclerosis and fibrillary glomerulonephritis. Complications included volume overload and congestive heart failure.

HERCEPTIN® (Trastuzumab)

Table 4
Adverse Events Occurring in ≥5% of Patients or at Increased Incidence in the HERCEPTIN Arm of the Randomized Study (Percent of Patients)

	Single Agent n=352	HERCEPTIN + Paclitaxel n=91	Paclitaxel Alone n=95	HERCEPTIN + AC n=143	AC Alone n=135
Body as a Whole					
Pain	47	61	62	57	42
Asthenia	42	62	57	54	55
Fever	36	49	23	56	34
Chills	32	41	4	35	11
Headache	26	36	28	44	31
Abdominal pain	22	34	22	23	18
Back pain	22	34	30	27	15
Infection	20	47	27	47	31
Flu syndrome	10	12	5	12	6
Accidental injury	6	13	3	9	4
Allergic reaction	3	8	2	4	2
Cardiovascular					
Tachycardia	5	12	4	10	5
Congestive heart failure	7	11	1	28	7
Digestive					
Nausea	33	51	9	76	77
Diarrhea	25	45	29	45	26
Vomiting	23	37	28	53	49
Nausea and vomiting	8	14	11	18	9
Anorexia	14	24	16	31	26
Heme & Lymphatic					
Anemia	4	14	9	36	26
Leukopenia	3	24	17	52	34
Metabolic					
Peripheral edema	10	22	20	20	17
Edema	8	10	8	11	5
Musculoskeletal					
Bone pain	7	24	18	7	7
Arthralgia	6	37	21	8	9
Nervous					
Insomnia	14	25	13	29	15
Dizziness	13	22	24	24	18
Paresthesia	9	48	39	17	11
Depression	6	12	13	20	12
Peripheral neuritis	2	23	18	2	2
Neuropathy	1	13	5	4	4
Respiratory					
Cough increased	26	41	22	43	29
Dyspnea	22	27	26	42	25
Rhinitis	14	22	5	22	16
Pharyngitis	12	22	14	30	18
Sinusitis	9	21	7	13	6
Skin					
Rash	18	38	18	27	17
Herpes simplex	2	12	3	7	9
Acne	2	11	3	3	<1
Urogenital					
Urinary tract infection	5	18	14	13	7

HERCEPTIN® (Trastuzumab)

Other Serious Adverse Events

The following other serious adverse events occurred in at least one of the 958 patients treated with HERCEPTIN in clinical studies:

Body as a Whole: cellulitis, anaphylactoid reaction, ascites, hydrocephalus, radiation injury, deafness, amblyopia

Cardiovascular: vascular thrombosis, pericardial effusion, heart arrest, hypotension, syncope, hemorrhage, shock, arrhythmia

Digestive: hepatic failure, gastroenteritis, hematemesis, ileus, intestinal obstruction, colitis, esophageal ulcer, stomatitis, pancreatitis, hepatitis

Endocrine: hypothyroidism

Hematological: pancytopenia, acute leukemia, coagulation disorder, lymphangitis

Metabolic: hypercalcemia, hypomagnesemia, hyponatremia, hypoglycemia, growth retardation, weight loss

Musculoskeletal: pathological fractures, bone necrosis, myopathy

Nervous: convulsion, ataxia, confusion, manic reaction

Respiratory: apnea, pneumothorax, asthma, hypoxia, laryngitis

Skin: herpes zoster, skin ulceration

Urogenital: hydronephrosis, kidney failure, cervical cancer, hematuria, hemorrhagic cystitis, pyelonephritis

Immunogenicity:

Of 903 patients who have been evaluated, human anti-human antibody (HAHA) to Trastuzumab was detected in one patient, who had no allergic manifestations.

The data reflect the percentage of patients whose test results were considered positive for antibodies to HERCEPTIN in the HAHA assay for Trastuzumab, and are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors including sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to HERCEPTIN with the incidence of antibodies to other products may be misleading.

OVERDOSAGE

There is no experience with overdosage in human clinical trials. Single doses higher than 500 mg have not been tested.

DOSAGE AND ADMINISTRATION

Usual Dose

The recommended initial loading dose is 4 mg/kg Trastuzumab administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg Trastuzumab and can be administered as a 30-minute infusion if the initial loading dose was well tolerated. HERCEPTIN may be administered in an outpatient setting. HERCEPTIN is to be diluted in saline for IV infusion. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS.** (See DOSAGE AND ADMINISTRATION: Administration.)

Preparation for Administration

The diluent provided has been formulated to maintain the stability and sterility of HERCEPTIN for up to 28 days. Other diluents have not been shown to contain effective preservatives for HERCEPTIN. Each vial of HERCEPTIN should be reconstituted with 20 mL of BWFI, USP, 1.1% benzyl alcohol preserved, as supplied, to yield a multi-dose solution containing 21 mg/mL Trastuzumab. Immediately upon reconstitution with BWFI, the vial of HERCEPTIN must be labeled in the area marked "Do not use after:" with the future date that is 28 days from the date of reconstitution.

If the patient has known hypersensitivity to benzyl alcohol, HERCEPTIN must be reconstituted with Sterile Water for Injection. (See PRECAUTIONS.) HERCEPTIN WHICH HAS BEEN RECONSTITUTED WITH SWFI MUST BE USED IMMEDIATELY AND ANY UNUSED PORTION DISCARDED. USE OF OTHER RECONSTITUTION DILUENTS SHOULD BE AVOIDED.

Shaking the reconstituted HERCEPTIN or causing excessive foaming during the addition of diluent may result in problems with dissolution and the amount of HERCEPTIN that can be withdrawn from the vial.

Use appropriate aseptic technique when performing the following reconstitution steps:

- Using a sterile syringe, slowly inject the 20 mL of diluent into the vial containing the lyophilized cake of Trastuzumab. The stream of diluent should be directed into the lyophilized cake.
- Swirl the vial gently to aid reconstitution. Trastuzumab may be sensitive to shear-induced stress, e.g., agitation or rapid expulsion from a syringe. **DO NOT SHAKE.**
- Slight foaming of the product upon reconstitution is not unusual. Allow the vial to stand undisturbed for approximately 5 minutes. The solution should be essentially free of visible particulates, clear to slightly opalescent and colorless to pale yellow.

Determine the number of mg of Trastuzumab needed, based on a loading dose of 4 mg Trastuzumab/kg body weight or a maintenance dose of 2 mg Trastuzumab/kg body weight. Calculate the volume of 21 mg/mL Trastuzumab solution and withdraw this amount from the vial and add it to an infusion bag containing 250 mL of 0.9% Sodium Chloride Injection, USP. **DEXTROSE (5%) SOLUTION SHOULD NOT BE USED.** Gently invert the bag to mix the solution. The reconstituted preparation results in a colorless to pale yellow transparent solution. Parenteral drug products should be inspected visually for particulates and discoloration prior to administration.

No incompatibilities between HERCEPTIN and polyvinylchloride or polyethylene bags have been observed.

Administration

Treatment may be administered in an outpatient setting by administration of a 4 mg/kg Trastuzumab loading dose by intravenous (IV) infusion over 90 minutes. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS.** Patients should be observed for fever and chills or other infusion-associated symptoms. (See BOXED WARNINGS, WARNINGS, and ADVERSE REACTIONS.) If prior infusions are well tolerated, subsequent weekly doses of 2 mg/kg Trastuzumab may be administered over 30 minutes.

HERCEPTIN should not be mixed or diluted with other drugs. HERCEPTIN infusions should not be administered or mixed with Dextrose solutions.

Stability and Storage

Vials of HERCEPTIN are stable at 2–8°C (36–46°F) prior to reconstitution. Do not use beyond the expiration date stamped on the vial. A vial of HERCEPTIN reconstituted with BWFI, as supplied, is stable for 28 days after reconstitution when stored refrigerated at 2–8°C (36–46°F), and the solution is preserved for multiple use. Discard any remaining multi-dose reconstituted solution after 28 days. If unpreserved SWFI (not supplied) is used, the reconstituted HERCEPTIN solution should be used immediately and any unused portion must be discarded. **DO NOT FREEZE HERCEPTIN THAT HAS BEEN RECONSTITUTED.**

The solution of HERCEPTIN for infusion diluted in polyvinylchloride or polyethylene bags containing 0.9% Sodium Chloride Injection, USP, may be stored at 2–8°C (36–46°F) for up to 24 hours prior to use. Diluted HERCEPTIN has been shown to be stable for up to 24 hours at room temperature (2–25°C). However, since diluted HERCEPTIN contains no effective preservative, the reconstituted and diluted solution should be stored refrigerated (2–8°C).

HERCEPTIN® (Trastuzumab)

HOW SUPPLIED

HERCEPTIN is supplied as a lyophilized, sterile powder nominally containing 440 mg Trastuzumab per vial under vacuum.

Each carton contains one vial of 440 mg HERCEPTIN® (Trastuzumab) and one vial containing 20 mL of Bacteriostatic Water for Injection, USP, 1.1% benzyl alcohol. NDC 50242-134-68.

REFERENCES

- Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, McGrath J, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. *Science* 1985;230:1132–9.
- Press MF, Pike MC, Chazin VR, Hung G, Udoe JA, Markowicz M, et al. *Her-2/neu* expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. *Cancer Res* 1993;53:4960–70.
- Stamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the *HER2/neu* proto-oncogene in human breast and ovarian cancer. *Science* 1989;244:707–12.
- Kallioniemi OP, Kallioniemi A, Kurisu W, Thor A, Chen L-C, Smith HS, et al. *ERBB2* amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc Natl Acad Sci USA* 1992;89:5321–5.
- Pauletti G, Godolphin W, Press MF, Stamon DJ. Detection and quantitation of *HER2/neu* gene amplification in human breast cancer archival material using fluorescence in situ hybridization. *Oncogene* 1996;13:63–72.
- Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. p185^{HER2} monoclonal antibody has antiproliferative effects *in vitro* and sensitizes human breast tumor cells to tumor necrosis factor. *Mol Cell Biol* 1989;9:1165–72.
- Lewis GD, Figari I, Fendly B, Wong WL, Carter P, Gorman C, et al. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. *Cancer Immunol Immunother* 1993;37:255–63.
- Baselga J, Norton L, Albanell J, Kim Y-M, Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herceptin®) enhances the antitumor activity of paclitaxel and doxorubicin against *HER2/neu* overexpressing human breast cancer xenografts. *Cancer Res* 1998;58:2825–31.
- Hotaling TE, Reitz B, Wolfgang-Kimball D, Bauer K, Fox JA. The humanized anti-HER2 antibody huMAb HER2 mediates antibody dependent cell-mediated cytotoxicity via FcγR III [abstract]. *Proc Annu Meet Am Assoc Cancer Res* 1996;37:471.
- Pegram MD, Baly D, Wirth C, Glikerson E, Stamon DJ, Siwkowski MX, et al. Antibody dependent cell-mediated cytotoxicity in breast cancer patients in Phase III clinical trials of a humanized anti-HER2 antibody [abstract]. *Proc Am Assoc Cancer Res* 1997;38:602.
- Stamon D J., Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344:783–92.
- Lee, KS. Requirement for neuroregulin receptor, erbB2, in neural and cardiac development. *Nature* 1995;379:394–6.

HERCEPTIN®

(Trastuzumab)

Manufactured by:

Genentech, Inc.

1 DNA Way

South San Francisco, CA 94080-4990

LK0141

7172904

(4817404)

FDA revision August 2002

Code revision August 2002

©2002 Genentech, Inc.

HERCEPTIN® (Trastuzumab)

Other Serious Adverse Events

The following other serious adverse events occurred in at least one of the 958 patients treated with HERCEPTIN in clinical studies:

Body as a Whole: cellulitis, anaphylactoid reaction, ascites, hydrocephalus, radiation injury, deafness, amblyopia

Cardiovascular: vascular thrombosis, pericardial effusion, heart arrest, hypotension, syncope, hemorrhage, shock, arrhythmia

Digestive: hepatic failure, gastroenteritis, hematemesis, ileus, intestinal obstruction, colitis, esophageal ulcer, stomatitis, pancreatitis, hepatitis

Endocrine: hypothyroidism

Hematological: pancytopenia, acute leukemia, coagulation disorder, lymphangitis

Metabolic: hypercalcemia, hypomagnesemia, hyponatremia, hypoglycemia, growth retardation, weight loss

Musculoskeletal: pathological fractures, bone necrosis, myopathy

Nervous: convulsion, ataxia, confusion, manic reaction

Respiratory: apnea, pneumothorax, asthma, hypoxia, laryngitis

Skin: herpes zoster, skin ulceration

Urogenital: hydronephrosis, kidney failure, cervical cancer, hematuria, hemorrhagic cystitis, pyelonephritis

Immunogenicity:

Of 903 patients who have been evaluated, human anti-human antibody (HAHA) to Trastuzumab was detected in one patient, who had no allergic manifestations.

The data reflect the percentage of patients whose test results were considered positive for antibodies to HERCEPTIN in the HAHA assay for Trastuzumab, and are highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors including sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies to HERCEPTIN with the incidence of antibodies to other products may be misleading.

OVERDOSAGE

There is no experience with overdosage in human clinical trials. Single doses higher than 500 mg have not been tested.

DOSAGE AND ADMINISTRATION

Usual Dose

The recommended initial loading dose is 4 mg/kg Trastuzumab administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg Trastuzumab and can be administered as a 30-minute infusion if the initial loading dose was well tolerated. HERCEPTIN may be administered in an outpatient setting. HERCEPTIN is to be diluted in saline for IV infusion. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS.** (See DOSAGE AND ADMINISTRATION: Administration.)

Preparation for Administration

The diluent provided has been formulated to maintain the stability and sterility of HERCEPTIN for up to 28 days. Other diluents have not been shown to contain effective preservatives for HERCEPTIN. Each vial of HERCEPTIN should be reconstituted with 20 mL of BWF, USP, 1.1% benzyl alcohol preserved, as supplied, to yield a multi-dose solution containing 21 mg/mL Trastuzumab. Immediately upon reconstitution with BWF, the vial of HERCEPTIN must be labeled in the area marked "Do not use after:" with the future date that is 28 days from the date of reconstitution.

If the patient has known hypersensitivity to benzyl alcohol, HERCEPTIN must be reconstituted with Sterile Water for Injection. (See PRECAUTIONS.) HERCEPTIN WHICH HAS BEEN RECONSTITUTED WITH SWFI MUST BE USED IMMEDIATELY AND ANY UNUSED PORTION DISCARDED. USE OF OTHER RECONSTITUTION DILUENTS SHOULD BE AVOIDED.

Shaking the reconstituted HERCEPTIN or causing excessive foaming during the addition of diluent may result in problems with dissolution and the amount of HERCEPTIN that can be withdrawn from the vial.

Use appropriate aseptic technique when performing the following reconstitution steps:

- Using a sterile syringe, slowly inject the 20 mL of diluent into the vial containing the lyophilized cake of Trastuzumab. The stream of diluent should be directed into the lyophilized cake.
- Swirl the vial gently to aid reconstitution. Trastuzumab may be sensitive to shear-induced stress, e.g., agitation or rapid expulsion from a syringe. **DO NOT SHAKE.**
- Slight foaming of the product upon reconstitution is not unusual. Allow the vial to stand undisturbed for approximately 5 minutes. The solution should be essentially free of visible particulates, clear to slightly opalescent and colorless to pale yellow.

Determine the number of mg of Trastuzumab needed, based on a loading dose of 4 mg Trastuzumab/kg body weight or a maintenance dose of 2 mg Trastuzumab/kg body weight. Calculate the volume of 21 mg/mL Trastuzumab solution and withdraw this amount from the vial and add it to an infusion bag containing 250 mL of 0.9% Sodium Chloride Injection, USP. **DEXTROSE (5%) SOLUTION SHOULD NOT BE USED.** Gently invert the bag to mix the solution. The reconstituted preparation results in a colorless to pale yellow transparent solution. Parenteral drug products should be inspected visually for particulates and discoloration prior to administration.

No incompatibilities between HERCEPTIN and polyvinylchloride or polyethylene bags have been observed.

Administration

Treatment may be administered in an outpatient setting by administration of a 4 mg/kg Trastuzumab loading dose by intravenous (IV) infusion over 90 minutes. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS.** Patients should be observed for fever and chills or other infusion-associated symptoms. (See BOXED WARNINGS, WARNINGS, and ADVERSE REACTIONS.) If prior infusions are well tolerated, subsequent weekly doses of 2 mg/kg Trastuzumab may be administered over 30 minutes.

HERCEPTIN should not be mixed or diluted with other drugs. HERCEPTIN infusions should not be administered or mixed with Dextrose solutions.

Stability and Storage

Vials of HERCEPTIN are stable at 2–8°C (36–46°F) prior to reconstitution. Do not use beyond the expiration date stamped on the vial. A vial of HERCEPTIN reconstituted with BWF, as supplied, is stable for 28 days after reconstitution when stored refrigerated at 2–8°C (36–46°F), and the solution is preserved for multiple use. Discard any remaining multi-dose reconstituted solution after 28 days. If unpreserved SWFI (not supplied) is used, the reconstituted HERCEPTIN solution should be used immediately and any unused portion must be discarded. **DO NOT FREEZE HERCEPTIN THAT HAS BEEN RECONSTITUTED.**

The solution of HERCEPTIN for infusion diluted in polyvinylchloride or polyethylene bags containing 0.9% Sodium Chloride Injection, USP, may be stored at 2–8°C (36–46°F) for up to 24 hours prior to use. Diluted HERCEPTIN has been shown to be stable for up to 24 hours at room temperature (2–25°C). However, since diluted HERCEPTIN contains no effective preservative, the reconstituted and diluted solution should be stored refrigerated (2–8°C).

HERCEPTIN® (Trastuzumab)

HOW SUPPLIED

HERCEPTIN is supplied as a lyophilized, sterile powder nominally containing 440 mg Trastuzumab per vial under vacuum.

Each carton contains one vial of 440 mg HERCEPTIN® (Trastuzumab) and one vial containing 20 mL of Bacteriostatic Water for Injection, USP, 1.1% benzyl alcohol. NDC 50242-134-68.

REFERENCES

- Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, McGrath J, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with new oncogene. *Science* 1985;230:1132–9.
- Press MF, Pike MC, Chazin VR, Hung G, Udove JA, Markowitz M, et al. Her-2/*neu* expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. *Cancer Res* 1993;53:4960–70.
- Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER2/*neu* proto-oncogene in human breast and ovarian cancer. *Science* 1989;244:707–12.
- Kallioniemi OP, Kallioniemi A, Kurisu W, Thor A, Chen L-C, Smith HS, et al. *ERBB2* amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc Natl Acad Sci USA* 1992;89:5321–5.
- Pauletti G, Godolphin W, Press MF, Slamon DJ. Detection and quantitation of HER2/*neu* gene amplification in human breast cancer archival material using fluorescence in situ hybridization. *Oncogene* 1996;13:63–72.
- Hudisak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. p185^{HER2} monoclonal antibody has antiproliferative effects *in vitro* and sensitizes human breast tumor cells to tumor necrosis factor. *Mol Cell Biol* 1989;9:1165–72.
- Lewis GD, Figari I, Fendly B, Wong WL, Carter P, Gorman C, et al. Differential responses of human tumor cell lines to anti-p185HER2 monoclonal antibodies. *Cancer Immunol Immunother* 1993;37:255–63.
- Baselga J, Norton L, Albanell J, Kim Y-M, Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/*neu* overexpressing human breast cancer xenografts. *Cancer Res* 1998;58:2825–31.
- Hotaling TE, Reitz B, Wolfgang-Kimball D, Bauer K, Fox JA. The humanized anti-HER2 antibody rhuMAB HER2 mediates antibody dependent cell-mediated cytotoxicity via FcγR III [abstract]. *Proc Annu Meet Am Assoc Cancer Res* 1996;37:471.
- Pegram MD, Baly D, Wirth C, Glikerson E, Slamon DJ, Sitkowski MX, et al. Antibody dependent cell-mediated cytotoxicity in breast cancer patients in Phase III clinical trials of a humanized anti-HER2 antibody [abstract]. *Proc Am Assoc Cancer Res* 1997;38:602.
- Slamon D J., Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med*. 2001;344:783–92.
- Lee, KS. Requirement for neuroregulin receptor, erbB2, in neural and cardiac development. *Nature* 1995;379:394–6.

HERCEPTIN®

(Trastuzumab)

Manufactured by:

Genentech, Inc.

1 DNA Way

South San Francisco, CA 94080-4990

LK0141

7172904

(4817404)

FDA revision August 2002

Code revision August 2002

©2002 Genentech, Inc.

**U.S. Food and Drug Administration****CENTER FOR DRUG EVALUATION AND RESEARCH**[FDA Home Page](#) | [CDER Home Page](#) | [CDER Site Info](#) | [Contact CDER](#) | [What's New @ CDER](#)

**Package Insert
Herceptin (Trastuzumab)
Genetech, Inc.**

Table of Contents

[Description](#)
[Clinical Pharmacology](#)
[Clinical Studies](#)
[Indications and Usage](#)
[Contraindications](#)
[Warnings](#)
[Precautions](#)
[Adverse Reactions](#)
[Overdosage](#)
[Dosage and Administration](#)
[How Supplied](#)
[References](#)

WARNING**CARDIOMYOPATHY:**

HERCEPTIN administration can result in the development of ventricular dysfunction and congestive heart failure. Left ventricular function should be evaluated in all patients prior to and during treatment with HERCEPTIN. Discontinuation of HERCEPTIN treatment should be strongly considered in patients who develop a clinically significant decrease in left ventricular function. The incidence and severity of cardiac dysfunction was particularly high in patients who received HERCEPTIN in combination with anthracyclines and cyclophosphamide. (See **WARNINGS**.)

DESCRIPTION

HERCEPTIN (Trastuzumab) is a recombinant DNA-derived humanized monoclonal antibody that selectively binds with high affinity in a cell-based assay ($K_d = 5 \text{ nM}$) to the extracellular domain of the human epidermal growth factor receptor 2 protein, HER2.^{1,2} The antibody is an IgG₁ kappa that contains human framework regions with the complementarity-determining regions of a murine antibody (4D5) that binds to HER2.

The humanized antibody against HER2 is produced by a mammalian cell (Chinese Hamster Ovary)

[CHO] suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product.

HERCEPTIN is a sterile, white to pale yellow, preservative-free lyophilized powder for intravenous (IV) administration. The nominal content of each HERCEPTIN vial is 440 mg Trastuzumab, 9.9 mg L-histidine HCl, 6.4 mg L-histidine, 400 mg α,α -trehalose dihydrate, and 1.8 mg polysorbate 20, USP. Reconstitution with **only 20 mL of the supplied Bacteriostatic Water for Injection (BWFJ), USP**, containing 1.1% benzyl alcohol as a preservative, yields a multi-dose solution containing 21 mg/mL Trastuzumab, at a pH of approximately 6.

[Return to Table of Contents](#)

CLINICAL PHARMACOLOGY

General

The HER2 (or c-erbB2) proto-oncogene encodes a transmembrane receptor protein of 185 kDa, which is structurally related to the epidermal growth factor receptor.¹ HER2 protein overexpression is observed in 25%-30% of primary breast cancers. HER2 protein overexpression can be determined using an immunohistochemistry-based assessment of fixed tumor blocks.³

Trastuzumab has been shown, in both *in vitro* assays and in animals, to inhibit the proliferation of human tumor cells that overexpress HER2.⁴⁻⁶

Trastuzumab is a mediator of antibody-dependent cellular cytotoxicity (ADCC).^{7,8} *In vitro*, HERCEPTIN-mediated ADCC has been shown to be preferentially exerted on HER2 overexpressing cancer cells compared with cancer cells that do not overexpress HER2.

[Return to Table of Contents](#)

Pharmacokinetics

The pharmacokinetics of Trastuzumab were studied in breast cancer patients with metastatic disease. Short duration intravenous infusions of 10 to 500 mg once weekly demonstrated dose-dependent pharmacokinetics. Mean half-life increased and clearance decreased with increasing dose level. The half-life averaged 1.7 and 12 days at the 10 and 500 mg dose levels, respectively. Trastuzumab's volume of distribution was approximately that of serum volume (44 mL/kg). At the highest weekly dose studied (500 mg), mean peak serum concentrations were 377 microgram/mL.

In studies using a loading dose of 4 mg/kg followed by a weekly maintenance dose of 2 mg/kg, a mean half-life of 5.8 days (range = 1 to 32 days) was observed. Between Weeks 16 and 32, Trastuzumab serum concentrations reached a steady state with a mean trough and peak concentrations of approximately 79 microgram/mL and 123 microgram/mL, respectively.

Detectable concentrations of the circulating extracellular domain of the HER2 receptor (shed antigen) are found in the serum of some patients with HER2 overexpressing tumors. Determination of shed antigen in baseline serum samples revealed that 64% (286/447) of patients had detectable shed antigen, which ranged as high as 1880 ng/mL (median = 11 ng/mL). Patients with higher baseline

shed antigen levels were more likely to have lower serum trough concentrations. However, with weekly dosing, most patients with elevated shed antigen levels achieved target serum concentrations of Trastuzumab by Week 6.

Data suggest that the disposition of Trastuzumab is not altered based on age or serum creatinine (up to 2.0 mg/dL). No formal interaction studies have been performed.

Mean serum trough concentrations of Trastuzumab, when administered in combination with paclitaxel, were consistently elevated approximately 1.5-fold as compared with serum concentrations of Trastuzumab used in combination with anthracycline plus cyclophosphamide. In primate studies, administration of Trastuzumab with paclitaxel resulted in a reduction in Trastuzumab clearance. Serum levels of Trastuzumab in combination with cisplatin, doxorubicin or epirubicin plus cyclophosphamide did not suggest any interactions; no formal drug interaction studies were performed.

[Return to Table of Contents](#)

CLINICAL STUDIES

The safety and efficacy of HERCEPTIN were studied in a randomized, controlled clinical trial in combination with chemotherapy (469 patients) and an open-label single agent clinical trial (222 patients). Both trials studied patients with metastatic breast cancer whose tumors overexpress the HER2 protein. Patients were eligible if they had 2+ or 3+ levels of overexpression (based on a 0-3+ scale) by immunohistochemical assessment of tumor tissue performed by a central testing lab.

A multicenter, randomized, controlled clinical trial was conducted in 469 patients with metastatic breast cancer who had not been previously treated with chemotherapy for metastatic disease. Patients were randomized to receive chemotherapy alone or in combination with HERCEPTIN given intravenously as a 4 mg/kg loading dose followed by weekly doses of HERCEPTIN at 2 mg/kg. For those who had received prior anthracycline therapy in the adjuvant setting, chemotherapy consisted of paclitaxel (175 mg/m² over 3 hours every 21 days for at least six cycles); for all other patients, chemotherapy consisted of anthracycline plus cyclophosphamide (AC: doxorubicin 60 mg/m² or epirubicin 75 mg/m² plus 600 mg/m² cyclophosphamide every 21 days for six cycles). Compared with patients in the AC subgroups (n = 281), patients in the paclitaxel subgroups (n = 188) were more likely to have had the following: poor prognostic factors (premenopausal status, estrogen or progesterone receptor negative tumors, positive lymph nodes), prior therapy (adjuvant chemotherapy, myeloablative chemotherapy, radiotherapy), and a shorter disease-free interval.

Compared with patients randomized to chemotherapy alone, the patients randomized to HERCEPTIN and chemotherapy experienced a significantly longer median time to disease progression, a higher overall response rate (ORR), a longer median duration of response, and a higher one-year survival rate. (See Table 1.) These treatment effects were observed both in patients who received HERCEPTIN plus paclitaxel and in those who received HERCEPTIN plus AC, however the magnitude of the effects was greater in the paclitaxel subgroup. The degree of HER2 overexpression was a predictor of treatment effect. (See CLINICAL STUDIES: HER2 protein overexpression.)

Table 1
Phase III Clinical Efficacy in First-Line Treatment

	Combined Results		Paclitaxel subgroup		AC subgroup	
	HERCEPTIN + All Chemotherapy (n = 235)	All Chemotherapy (n = 234)	HERCEPTIN + Paclitaxel (n = 92)	Paclitaxel (n = 96)	HERCEPTIN + AC ^a (n = 143)	AC (n = 138)
Primary Endpoint						
Time to Progression ^{b, c}						
Median (months)	7.2	4.5	6.7	2.5	7.6	5.7
95% confidence interval	6.9, 8.2	4.3, 4.9	5.2, 9.9	2.0, 4.3	7.2, 9.1	4.6, 7.1
p-value (log rank)	<0.0001		<0.0001		0.002	
Secondary Endpoints						
Overall Response Rate ^b						
Rate (percent)	45	29	38	15	50	38
95% confidence interval	39, 51	23, 35	28, 48	8, 22	42, 58	30, 46
p-value (χ2-test)	<0.001		<0.001		0.10	
Duration of Response ^{b, c}						
Median (months)	8.3	5.8	8.3	4.3	8.4	6.4
25%, 75% quantile	5.5, 14.8	3.9, 8.5	5.1, 11.0	3.7, 7.4	5.8, 14.8	4.5, 8.5
1-Year Survival ^c						
Percent alive	79	68	73	61	83	73
95% confidence interval	74, 84	62, 74	66, 80	51, 71	77, 89	66, 82

p-value (Z-test)	<0.01	0.08	0.04
------------------	-------	------	------

^a AC = anthracycline (doxorubicin or epirubicin) and cyclophosphamide.

^b Assessed by an independent Response Evaluation Committee.

^c Kaplan-Meier Estimate

HERCEPTIN was studied as a single agent in a multicenter, open-label, single-arm clinical trial in patients with HER2 overexpressing metastatic breast cancer who had relapsed following one or two prior chemotherapy regimens for metastatic disease. Of 222 patients enrolled, 66% had received prior adjuvant chemotherapy, 68% had received two prior chemotherapy regimens for metastatic disease, and 25% had received prior myeloablative treatment with hematopoietic rescue. Patients were treated with a loading dose of 4 mg/kg IV followed by weekly doses of HERCEPTIN at 2 mg/kg IV. The ORR (complete response + partial response), as determined by an independent Response Evaluation Committee, was 14%, with a 2% complete response rate and a 12% partial response rate. Complete responses were observed only in patients with disease limited to skin and lymph nodes. The degree of HER2 overexpression was a predictor of treatment effect. (See CLINICAL STUDIES: HER2 protein overexpression.)

[Return to Table of Contents](#)

HER2 Protein Overexpression

Relationship to Response: In the clinical studies described, patient eligibility was determined by testing tumor specimens for overexpression of HER2 protein. Specimens were tested with a research-use-only immunohistochemical assay (referred to as the Clinical Trial Assay, CTA) and scored as 0, 1+, 2+, or 3+ with 3+ indicating the strongest positivity. Only patients with 2+ or 3+ positive tumors were eligible (about 33% of those screened).

Data from both efficacy trials suggest that the beneficial treatment effects were largely limited to patients with the highest level of HER2 protein overexpression (3+). (See Table 2.)

Table 2
Treatment Effect versus Level of HER2 Expression

	Single-Arm Trial	Treatment Subgroups in Randomized Trial			
	HERCEPTIN	HERCEPTIN + Paclitaxel	Paclitaxel	HERCEPTIN + AC	AC
Overall Response Rate					

2+ overexpression	4% (2/50)	21% (5/24)	16% (3/19)	40% (14/35)	43% (18/42)
3+ overexpression	17% (29/172)	44% (30/68)	14% (11/77)	53% (57/108)	36% (35/96)
Median time to progression (months) (95% CI)					
2+ overexpression	N/A ^a	4.4 (2.2, 6.6)	3.2 (2.0, 5.6)	7.8 (6.4, 10.1)	7.1 (4.8, 9.8)
3+ overexpression	N/A ^a	7.1 (6.2, 12.0)	2.2 (1.8, 4.3)	7.3 (7.1, 9.2)	4.9 (4.5, 6.9)

^a N/A = Not Assessed

Immunohistochemical Detection: In clinical trials, the Clinical Trial Assay (CTA) was used for immunohistochemical detection of HER2 protein overexpression. The DAKO HercepTest™, , another immunohistochemical test for HER2 protein overexpression, has not been directly studied for its ability to predict HERCEPTIN treatment effect, but has been compared to the CTA on over 500 breast cancer histology specimens obtained from the National Cancer Institute Cooperative Breast Cancer Tissue Resource. Based upon these results and an expected incidence of 33% of 2+ or 3+ HER2 overexpression in tumors from women with metastatic breast cancer, one can estimate the correlation of the HercepTest results with CTA results. Of specimens testing 3+ (strongly positive) on the HercepTest, 94% would be expected to test at least 2+ on the CTA (i.e., meeting the study entry criterion) including 82% which would be expected to test 3+ on the CTA (i.e., the reading most associated with clinical benefit). Of specimens testing 2+ (weakly positive) on the HercepTest, only 34% would be expected to test at least 2+ on the CTA, including 14% which would be expected to test 3+ on the CTA.

[Return to Table of Contents](#)

INDICATIONS AND USAGE

HERCEPTIN as a single agent is indicated for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have received one or more chemotherapy regimens for their metastatic disease. HERCEPTIN in combination with paclitaxel is indicated for treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have not received chemotherapy for their metastatic disease. HERCEPTIN should only be used in patients whose tumors have HER2 protein overexpression. (See [CLINICAL STUDIES: HER2 protein overexpression](#) for information regarding HER2 protein testing and the relationship between the degree of overexpression and the treatment effect.)

[Return to Table of Contents](#)

CONTRAINDICATIONS

None known.

[Return to Table of Contents](#)

WARNINGS

Cardiotoxicity:

Signs and symptoms of cardiac dysfunction, such as dyspnea, increased cough, paroxysmal nocturnal dyspnea, peripheral edema, S₃ gallop, or reduced ejection fraction, have been observed in patients treated with HERCEPTIN. Congestive heart failure associated with HERCEPTIN therapy may be severe and has been associated with disabling cardiac failure, death, and mural thrombosis leading to stroke. The clinical status of patients in the trials who developed congestive heart failure was classified for severity using the New York Heart Association classification system (I-IV, where IV is the most severe level of cardiac failure). (See Table 3.)

Table 3
Incidence and Severity of Cardiac Dysfunction

	HERCEPTIN ^a alone n = 213	HERCEPTIN + Paclitaxel ^b n = 91	Paclitaxel ^b n = 95	HERCEPTIN+ Anthracycline+ cyclophosphamide ^b n = 143	Anthracycline+ cyclophosphamide ^b n = 135
Any Cardiac Dysfunction	7%	11%	1%	28%	7%
Class III-IV	5%	4%	1%	19%	3%

^aOpen-label, single-agent Phase II study (94% received prior anthracyclines).

^bRandomized Phase III study comparing chemotherapy plus HERCEPTIN to chemotherapy alone, where chemotherapy is either anthracycline/cyclophosphamide or paclitaxel.

Candidates for treatment with HERCEPTIN should undergo thorough baseline cardiac assessment including history and physical exam and one or more of the following: EKG, echocardiogram, and MUGA scan. There are no data regarding the most appropriate method of evaluation for the identification of patients at risk for developing cardiotoxicity. Monitoring may not identify all patients who will develop cardiac dysfunction.

Extreme caution should be exercised in treating patients with pre-existing cardiac dysfunction.

Patients receiving HERCEPTIN should undergo frequent monitoring for deteriorating cardiac function.

The probability of cardiac dysfunction was highest in patients who received HERCEPTIN

concurrently with anthracyclines. The data suggest that advanced age may increase the probability of cardiac dysfunction.

Pre-existing cardiac disease or prior cardiotoxic therapy (e.g., anthracycline or radiation therapy to the chest) may decrease the ability to tolerate HERCEPTIN therapy; however, the data are not adequate to evaluate the correlation between HERCEPTIN-induced cardiotoxicity and these factors.

Discontinuation of HERCEPTIN therapy should be strongly considered in patients who develop clinically significant congestive heart failure. In the clinical trials, most patients with cardiac dysfunction responded to appropriate medical therapy often including discontinuation of HERCEPTIN. The safety of continuation or resumption of HERCEPTIN in patients who have previously experienced cardiac toxicity has not been studied. There are insufficient data regarding discontinuation of HERCEPTIN therapy in patients with asymptomatic decreases in ejection fraction; such patients should be closely monitored for evidence of clinical deterioration.

[Return to Table of Contents](#)

PRECAUTIONS

General: HERCEPTIN therapy should be used with caution in patients with known hypersensitivity to Trastuzumab, Chinese Hamster Ovary cell proteins, or any component of this product.

Drug Interactions: There have been no formal drug interaction studies performed with HERCEPTIN in humans. Administration of paclitaxel in combination with HERCEPTIN resulted in a two-fold decrease in HERCEPTIN clearance in a non-human primate study and in a 1.5-fold increase in HERCEPTIN serum levels in clinical studies. (See [PHARMACOKINETICS](#).)

Benzyl Alcohol: For patients with a known hypersensitivity to benzyl alcohol (the preservative in Bacteriostatic Water for Injection) reconstitute HERCEPTIN with Sterile Water for Injection (SWFI), USP. DISCARD THE SWFI-RECONSTITUTED HERCEPTIN VIAL FOLLOWING A SINGLE USE.

Immunogenicity: Of 903 patients who have been evaluated, human anti-human antibody (HAHA) to Trastuzumab was detected in one patient, who had no allergic manifestations.

Carcinogenesis, Mutagenesis, Impairment of Fertility:

Carcinogenesis: HERCEPTIN has not been tested for its carcinogenic potential.

Mutagenesis: No evidence of mutagenic activity was observed in Ames tests using six different test strains of bacteria, with and without metabolic activation, at concentrations of up to 5000 µg/mL Trastuzumab. Human peripheral blood lymphocytes treated *in vitro* at concentrations of up to 5000 µg/plate Trastuzumab, with and without metabolic activation, revealed no evidence of mutagenic potential. In an *in vivo* mutagenic assay (the micronucleus assay), no evidence of chromosomal damage to mouse bone marrow cells was observed following bolus intravenous doses of up to 118 mg/kg Trastuzumab.

Impairment of Fertility: A fertility study has been conducted in female cynomolgus monkeys at doses up to 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN and has revealed

no evidence of impaired fertility.

Pregnancy Category B: Reproduction studies have been conducted in cynomolgus monkeys at doses up to 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN and have revealed no evidence of impaired fertility or harm to the fetus. However, HER2 protein expression is high in many embryonic tissues including cardiac and neural tissues; in mutant mice lacking HER2, embryos died in early gestation.² Placental transfer of HERCEPTIN during the early (Days 20,50 of gestation) and late (Days 120,150 of gestation) fetal development period was observed in monkeys. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

Nursing Mothers: A study conducted in lactating cynomolgus monkeys at doses 25 times the weekly human maintenance dose of 2 mg/kg HERCEPTIN demonstrated that Trastuzumab is secreted in the milk. The presence of Trastuzumab in the serum of infant monkeys was not associated with any adverse effects on their growth or development from birth to 3 months of age. It is not known whether HERCEPTIN is excreted in human milk. Because human IgG is excreted in human milk, and the potential for absorption and harm to the infant is unknown, women should be advised to discontinue nursing during HERCEPTIN therapy and for 6 months after the last dose of HERCEPTIN.

Pediatric Use: The safety and effectiveness of HERCEPTIN in pediatric patients have not been established.

Geriatric Use: HERCEPTIN has been administered to 133 patients who were 65 years of age or over. The risk of cardiac dysfunction may be increased in geriatric patients. The reported clinical experience is not adequate to determine whether older patients respond differently from younger patients.

[Return to Table of Contents](#)

ADVERSE REACTIONS

A total of 958 patients have received HERCEPTIN alone or in combination with chemotherapy. Data in Table 4 are based on the experience with the recommended dosing regimen for HERCEPTIN in the randomized controlled clinical trial in 234 patients who received HERCEPTIN in combination with chemotherapy and four open-label studies of HERCEPTIN as a single agent in 352 patients at doses of 10-500 mg administered weekly.

Cardiac Failure/Dysfunction: For a description of cardiac toxicities, see [WARNINGS](#).

Anemia and Leukopenia: An increased incidence of anemia and leukopenia was observed in the treatment group receiving HERCEPTIN and chemotherapy, especially in the HERCEPTIN and AC subgroup, compared with the treatment group receiving chemotherapy alone. The majority of these cytopenic events were mild or moderate in intensity, reversible, and none resulted in discontinuation of therapy with HERCEPTIN.

Hematologic toxicity is infrequent following the administration of HERCEPTIN as a single agent, with an incidence of Grade III toxicities for WBC, platelets, hemoglobin all <1%. No Grade IV toxicities were observed.

Diarrhea: Of patients treated with HERCEPTIN as a single agent, 25% experienced diarrhea. An increased incidence of diarrhea, primarily mild to moderate in severity, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infection: An increased incidence of infections, primarily mild upper respiratory infections of minor clinical significance or catheter infections, was observed in patients receiving HERCEPTIN in combination with chemotherapy.

Infusion Reactions: During the first infusion with HERCEPTIN, a symptom complex most commonly consisting of chills and/or fever was observed in about 40% of patients in clinical trials. The symptoms were usually mild to moderate in severity and were treated with acetaminophen, diphenhydramine, and meperidine (with or without reduction in the rate of HERCEPTIN infusion). HERCEPTIN discontinuation was infrequent. Other signs and/or symptoms may include nausea, vomiting, pain (in some cases at tumor sites), rigors, headache, dizziness, dyspnea, hypotension, rash and asthenia. The symptoms occurred infrequently with subsequent HERCEPTIN infusions.

Table 4
Adverse Events Occurring in $\geq 5\%$ of Patients or at
Increased Incidence in the HERCEPTIN Arm of the Randomized Study
(Percent of Patients)

	Single Agent n = 352	HERCEPTIN + Paclitaxel n = 91	Paclitaxel Alone n = 95	HERCEPTIN + AC n = 143	AC Alone n = 135
Body as a Whole					
Pain	47	61	62	57	42
Asthenia	42	62	57	54	55
Fever	36	49	23	56	34
Chills	32	41	4	35	11
Headache	26	36	28	44	31
Abdominal pain	22	34	22	23	18
Back pain	22	34	30	27	15
Infection	20	47	27	47	31
Flu syndrome	10	12	5	12	6
Accidental injury	6	13	3	9	4
Allergic Reaction	3	8	2	4	2
Cardiovascular					
Tachycardia	5	12	4	10	5
Congestive heart failure	7	11	1	28	7

Digestive					
Nausea	33	51	9	76	77
Diarrhea	25	45	29	45	26
Vomiting	23	37	28	53	49
Nausea and vomiting	8	14	11	18	9
Anorexia	14	24	16	31	26
Heme & Lymphatic					
Anemia	4	14	9	36	26
Leukopenia	3	24	17	52	34
Metabolic					
Peripheral edema	10	22	20	20	17
Edema	8	10	8	11	5
Musculoskeletal					
Bone pain	7	24	18	7	7
Arthralgia	6	37	21	8	9
Nervous					
Insomnia	14	25	13	29	15
Dizziness	13	22	24	24	18
Paresthesia	9	48	39	17	11
Depression	6	12	13	20	12
Peripheral neuritis	2	23	16	2	2
Neuropathy	1	13	5	4	4
Respiratory					
Cough increased	26	41	22	43	29
Dyspnea	22	27	26	42	25
Rhinitis	14	22	5	22	16
Pharyngitis	12	22	14	30	18
Sinusitis	9	21	7	13	6
Skin					
Rash	18	38	18	27	17

Herpes simplex	2	12	3	7	9
Acne	2	11	3	3	<1
Urogenital					
Urinary tract infection	5	18	14	13	7

Other serious adverse events

The following other serious adverse events occurred in at least one of the 958 patients treated with HERCEPTIN:

Body as a Whole: cellulitis, anaphylactoid reaction, ascites, hydrocephalus, radiation injury, deafness, amblyopia

Cardiovascular: vascular thrombosis, pericardial effusion, heart arrest, hypotension, syncope, hemorrhage, shock, arrhythmia

Digestive: hepatic failure, gastroenteritis, hematemesis, ileus, intestinal obstruction, colitis, esophageal ulcer, stomatitis, pancreatitis, hepatitis

Endocrine: hypothyroidism

Hematological: pancytopenia, acute leukemia, coagulation disorder, lymphangitis

Metabolic: hypercalcemia, hypomagnesemia, hyponatremia, hypoglycemia, growth retardation, weight loss

Musculoskeletal: pathological fractures, bone necrosis, myopathy

Nervous: convulsion, ataxia, confusion, manic reaction

Respiratory: apnea, pneumothorax, asthma, hypoxia, laryngitis

Skin: herpes zoster, skin ulceration

Urogenital: hydronephrosis, kidney failure, cervical cancer, hematuria, hemorrhagic cystitis, pyelonephritis

[Return to Table of Contents](#)

OVERDOSAGE

There is no experience with overdosage in human clinical trials. Single doses higher than 500 mg have not been tested.

[Return to Table of Contents](#)

DOSAGE AND ADMINISTRATION

Usual Dose

The recommended initial loading dose is 4 mg/kg Trastuzumab administered as a 90-minute infusion. The recommended weekly maintenance dose is 2 mg/kg Trastuzumab and can be administered as a 30-minute infusion if the initial loading dose was well tolerated. HERCEPTIN may be administered in an outpatient setting. HERCEPTIN is to be diluted in saline for IV infusion. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS** (see [ADMINISTRATION](#)).

Preparation for Administration

The diluent provided has been formulated to maintain the stability and sterility of HERCEPTIN for up to 28 days. Other diluents have not been shown to contain effective preservatives for HERCEPTIN. Each vial of HERCEPTIN should be reconstituted with **ONLY 20 mL of BWFI, USP, 1.1% benzyl alcohol preserved, as supplied**, to yield a multi-dose solution containing 21 mg/mL Trastuzumab. Use of all 30 mL of diluent results in a lower-than-intended dose of HERCEPTIN. **THE REMAINDER (approximately 10 mL) OF THE DILUENT SHOULD BE DISCARDED.** Immediately upon reconstitution with BWFI, the vial of HERCEPTIN must be labeled in the area marked "Do not use after:" with the future date that is 28 days from the date of reconstitution.

If the patient has known hypersensitivity to benzyl alcohol, HERCEPTIN must be reconstituted with Sterile Water for Injection (see [PRECAUTIONS](#)). HERCEPTIN WHICH HAS BEEN RECONSTITUTED WITH SWFI MUST BE USED IMMEDIATELY AND ANY UNUSED PORTION DISCARDED. USE OF OTHER RECONSTITUTION DILUENTS SHOULD BE AVOIDED.

Shaking the reconstituted HERCEPTIN or causing excessive foaming during the addition of diluent may result in problems with dissolution and the amount of HERCEPTIN that can be withdrawn from the vial.

Use appropriate aseptic technique when performing the following reconstitution steps:

- a. Using a sterile syringe, slowly inject **20 mL** of the diluent into the vial containing the lyophilized cake of Trastuzumab. The stream of diluent should be directed into the lyophilized cake.
- b. Swirl the vial gently to aid reconstitution. Trastuzumab may be sensitive to shear-induced stress, e.g., agitation or rapid expulsion from a syringe. **DO NOT SHAKE.**
- c. Slight foaming of the product upon reconstitution is not unusual. Allow the vial to stand undisturbed for approximately 5 minutes. The solution should be essentially free of visible particulates, clear to slightly opalescent, and colorless to pale yellow.

Determine the number in mg of Trastuzumab needed, based on a loading dose of 4 mg Trastuzumab/kg body weight or a maintenance dose of 2 mg Trastuzumab/kg body weight. Calculate the volume of 21 mg/mL Trastuzumab solution and withdraw this amount from the vial and add it to an infusion bag containing 250 mL of 0.9% Sodium Chloride Injection, USP. **DEXTROSE (5%)**

SOLUTION SHOULD NOT BE USED. Gently invert the bag to mix the solution. The reconstituted preparation results in a colorless to pale yellow transparent solution. Parenteral drug products should be inspected visually for particulates and discoloration prior to administration.

No incompatibilities between HERCEPTIN and polyvinylchloride or polyethylene bags have been observed.

[Return to Table of Contents](#)

Administration

Treatment may be administered in an outpatient setting by administration of a 4 mg/kg Trastuzumab loading dose by intravenous (IV) infusion over 90 minutes. **DO NOT ADMINISTER AS AN IV PUSH OR BOLUS.** Patients should be observed for fever and chills or other infusion-associated symptoms (see [ADVERSE REACTIONS](#)). If prior infusions are well tolerated, subsequent weekly doses of 2 mg/kg Trastuzumab may be administered over 30 minutes.

HERCEPTIN should not be mixed or diluted with other drugs. HERCEPTIN infusions should not be administered or mixed with Dextrose solutions.

Stability and Storage

Vials of HERCEPTIN are stable at 2-8°C (36-46°F) prior to reconstitution. Do not use beyond the expiration date stamped on the vial. A vial of HERCEPTIN reconstituted with BWFI, as supplied, is stable for 28 days after reconstitution when stored refrigerated at 2-8°C (36-46°F), and the solution is preserved for multiple use. Discard any remaining multi-dose reconstituted solution after 28 days. If unpreserved SWFI (not supplied) is used, the reconstituted HERCEPTIN solution should be used immediately and any unused portion must be discarded. **DO NOT FREEZE HERCEPTIN THAT HAS BEEN RECONSTITUTED.**

The solution of HERCEPTIN for infusion diluted in polyvinylchloride or polyethylene bags containing 0.9% Sodium Chloride Injection, USP, may be stored at 2-8°C (36-46°F) for up to 24 hours prior to use. Diluted HERCEPTIN has been shown to be stable for up to 24 hours at room temperature (2-25°C). However, since diluted HERCEPTIN contains no effective preservative, the reconstituted and diluted solution should be stored refrigerated (2-8°C).

[Return to Table of Contents](#)

HOW SUPPLIED

HERCEPTIN is supplied as a lyophilized, sterile powder nominally containing 440 mg Trastuzumab per vial under vacuum.

Each carton contains one vial of 440 mg HERCEPTIN (Trastuzumab) and one 30 mL vial of Bacteriostatic Water for Injection, USP, 1.1% benzyl alcohol. NDC50242-134-60.

[Return to Table of Contents](#)

REFERENCES

1. Coussens L, Yang-Feng TL, Liao Y-C, Chen E, Gray A, McGrath J, et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. *Science* 1985; 230:1132-9.
2. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, et al. Studies of the HER2/*neu* proto-oncogene in human breast and ovarian cancer. *Science* 1989; 244:707-12.
3. Press MF, Pike MC, Chazin VR, Hung G, Udove JA, Markowicz M, et al. Her-2/*neu* expression in node-negative breast cancer: direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. *Cancer Res* 1993; 53:4960-70.
4. Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A. p185^{HER2} monoclonal antibody has antiproliferative effects *in vitro* and sensitizes human breast tumor cells to tumor necrosis factor. *Mol Cell Biol* 1989; 9:1165-72.
5. Lewis GD, Figari I, Fendly B, Wong WL, Carter P, Gorman C, et al. Differential responses of human tumor cell lines to anti-p185^{HER2} monoclonal antibodies. *Cancer Immunol Immunother* 1993; 37:255-63.
6. Baselga J, Norton L, Albanell J, Kim Y-M, Mendelsohn J. Recombinant humanized anti-HER2 antibody (HerceptinTM) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/*neu* overexpressing human breast cancer xenografts. *Cancer Res.* 1998; 58: 2825-31.
7. Hotaling TE, Reitz B, Wolfgang-Kimball D, Bauer K, Fox JA. The humanized anti-HER2 antibody rhuMAb HER2 mediates antibody dependent cell-mediated cytotoxicity via FcγR III [abstract]. Proc Annu Meet Am Assoc Cancer Res 1996; 37:471.
8. Pegram MD, Baly D, Wirth C, Gilkerson E, Slamon DJ, Sliwkowski MX, et al. Antibody dependent cell-mediated cytotoxicity in breast cancer patients in Phase III clinical trials of a humanized anti-HER2 antibody [abstract]. Proc Am Assoc Cancer Res 1997; 38:602.
9. Lee, KS. Requirement for neuroregulin receptor, erbB2, in neural and cardiac development. *Nature* 1995; 379: 394-6.

HERCEPTIN® (Trastuzumab)

Manufactured by:

Genentech, Inc.

1 DNA Way

South San Francisco, CA 94080-4990

©2000 Genentech, Inc.

7172901

(4817401 Revised January 2000)

LK0068

[Return to Table of Contents](#)

Last Updated: 12/10/2001

Date created: September 26, 2003

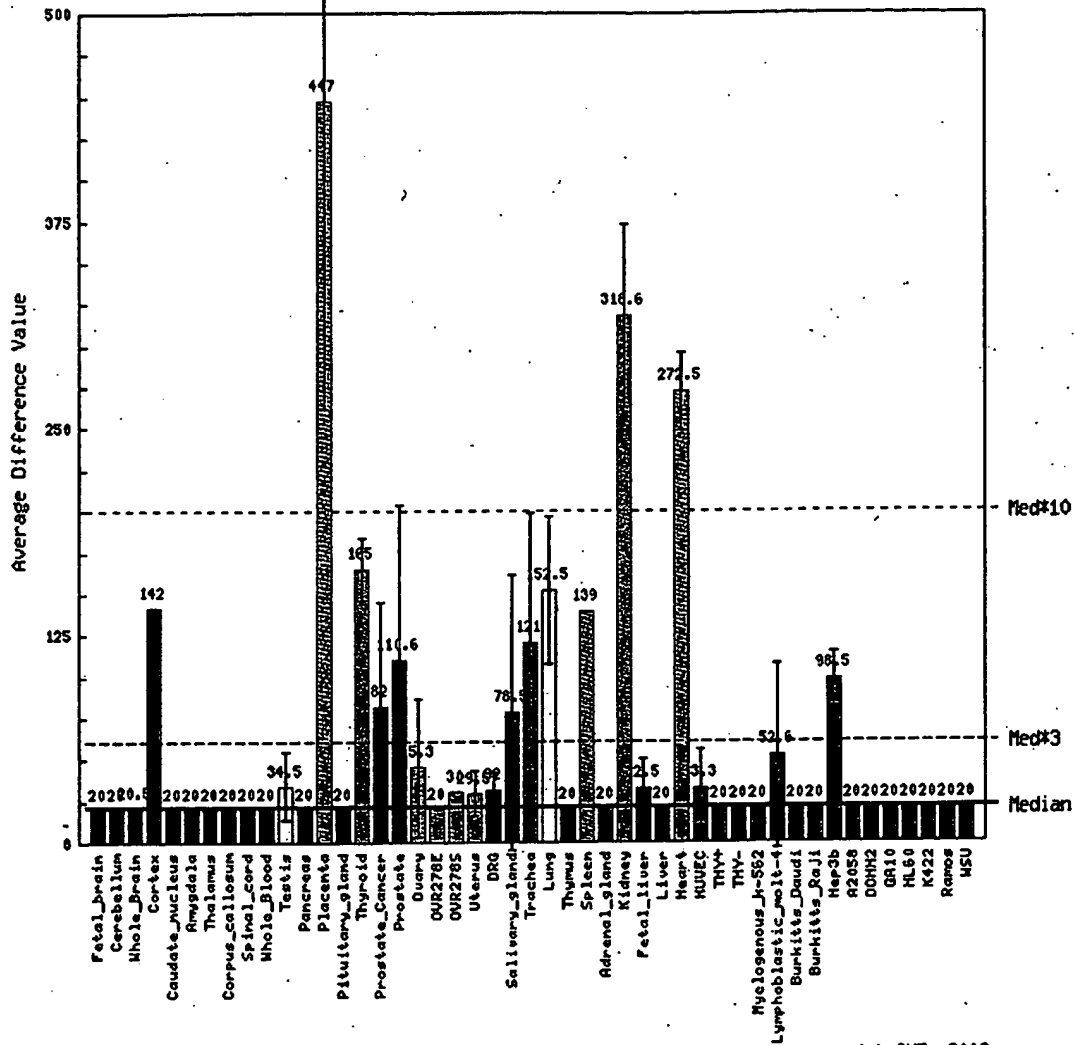
[CDER Home Page](#) | [CDER Site Info](#) | [Contact CDER](#) | [What's New @ CDER](#)
[FDA Home Page](#) | [Search FDA Site](#) | [FDA A-Z Index](#) | [Contact FDA](#) | [HHS Home Page](#)

FDA/Center for Drug Evaluation and Research

BEST AVAILABLE COPY

Hs_323910
ERBB2

v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (neuro/glioblastoma derived onco-
1901_s_at



(c) GNF, 2002

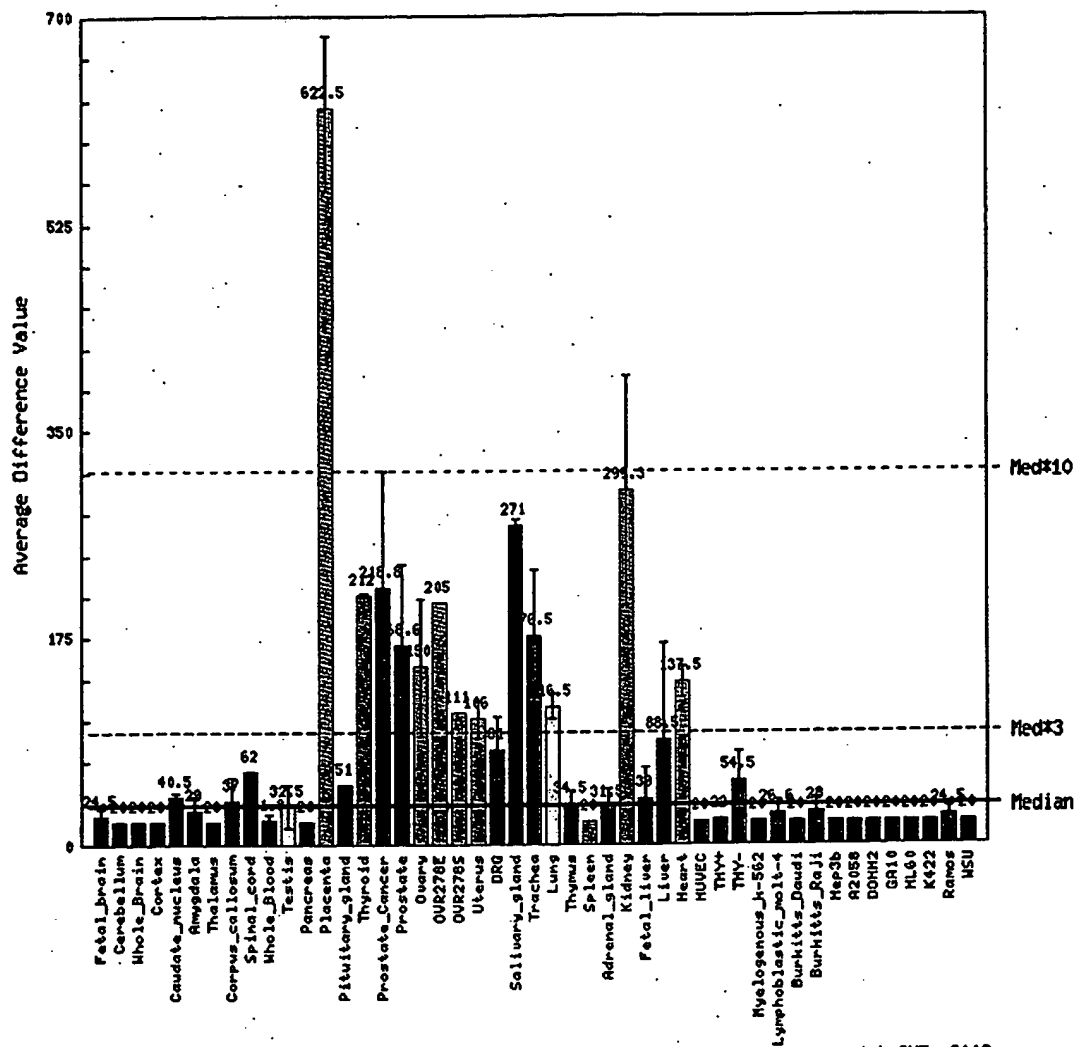
HER2/neu Tissue Expression
Based on analysis of GenBank Accession M12036

BEST AVAILABLE COPY

Hs.323910

ERBB2

v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (neuro/glioblastoma derived onco-
1802_s_at



(c) GNF, 2002

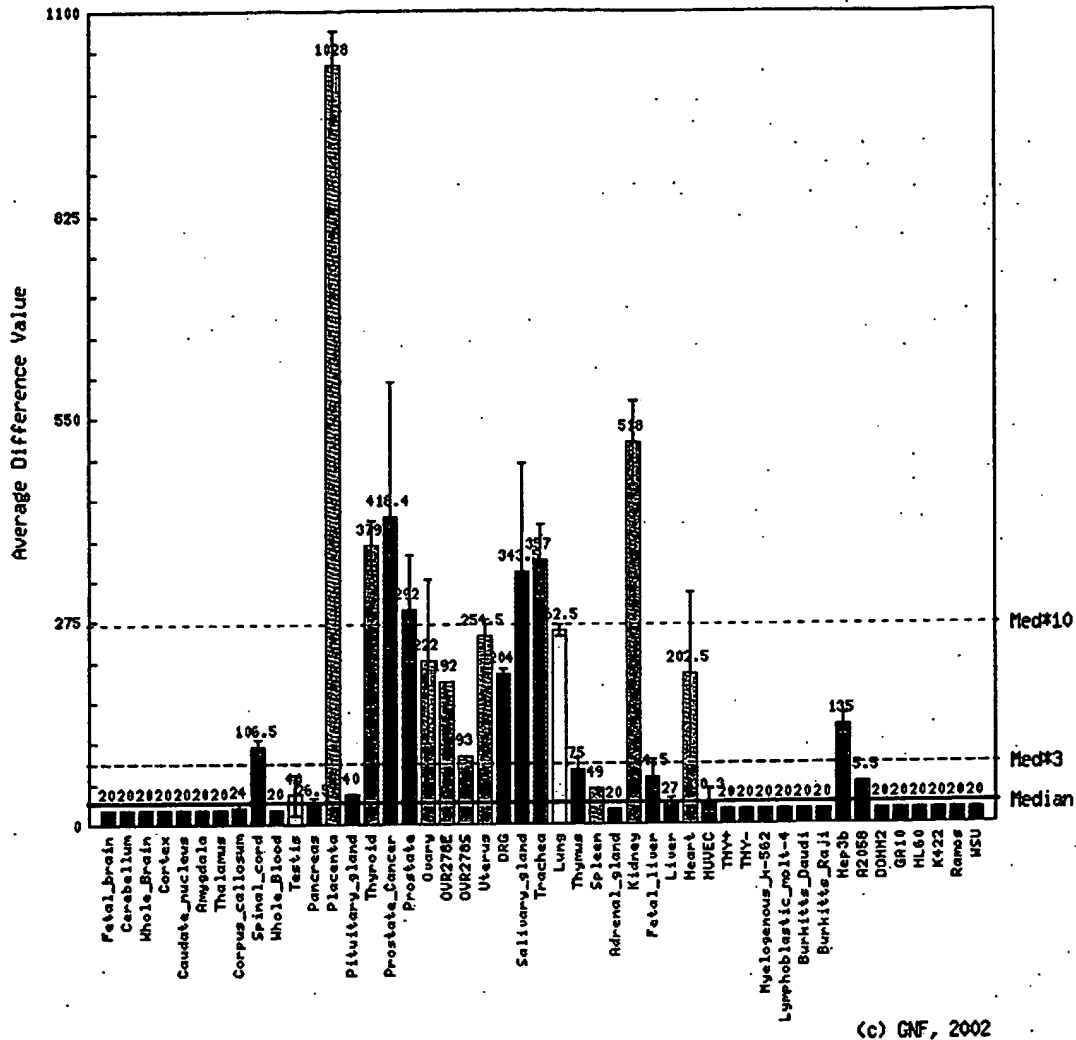
HER2/neu Tissue Expression
Based on analysis of GenBank Accession X03363

BEST AVAILABLE COPY

Hs.323910

ERBB2

v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (neuro/glioblastoma derived onco 33218_at



(c) GNF, 2002

HER2/neu Tissue Expression
Based on analysis of GenBank Accession M11730

Large-scale analysis of the human and mouse transcriptomes

Andrew I. Su^{*†}, Michael P. Cooke^{**}, Keith A. Ching^{**}, Yaron Hakak^{**}, John R. Walker^{**}, Tim Wiltshire^{**}, Anthony P. Orth^{**}, Raquel G. Vega^{*}, Lisa M. Sapinoso^{*}, Aziz Moqrich[‡], Ardem Patapoutian[‡], Garret M. Hampton^{*}, Peter G. Schultz^{**}, and John B. Hogenesch^{*‡}

Departments of ^{*}Chemistry and [‡]Cell Biology, The Scripps Research Institute, La Jolla, CA 92037; and ^{**}The Genomics Institute of the Novartis Research Foundation, San Diego, CA 92121-1125

Contributed by Peter G. Schultz, January 10, 2002

High-throughput gene expression profiling has become an important tool for investigating transcriptional activity in a variety of biological samples. To date, the vast majority of these experiments have focused on specific biological processes and perturbations. Here, we have generated and analyzed gene expression from a set of samples spanning a broad range of biological conditions. Specifically, we profiled gene expression from 91 human and mouse samples across a diverse array of tissues, organs, and cell lines. Because these samples predominantly come from the normal physiological state in the human and mouse, this dataset represents a preliminary, but substantial, description of the normal mammalian transcriptome. We have used this dataset to illustrate methods of mining these data, and to reveal insights into molecular and physiological gene function, mechanisms of transcriptional regulation, disease etiology, and comparative genomics. Finally, to allow the scientific community to use this resource, we have built a free and publicly accessible website (<http://expression.gnf.org>) that integrates data visualization and curation of current gene annotations.

The sequence of the first mammalian genome represents a landmark in modern biology and opens new avenues to pursue global approaches at understanding gene function and its relationship to human physiology (1, 2). The raw genome sequence and the accompanying gene predictions provide a starting point for the understanding of their function, the complexity of their interactions, and their roles in promoting cellular and organismal phenotypes. The most common approach to global gene annotation uses primary amino acid sequence analysis tools (e.g., BLAST and HMMER) and sequence databases (e.g., GenBank and Pfam; refs. 3–6). These powerful tools are used to annotate genes of unknown function under the premise that proteins of similar structure usually have similar function (e.g., kinases contain kinase domains).

Whereas primary sequence analysis frequently indicates the molecular function of a gene and can point to relevant biochemical assays for future study, it does not suggest the cellular or physiological role for proteins. To attempt to gain a more complete picture of a novel gene's function, researchers often perform multiple-tissue Northern blots to look at its expression in a panel of tissues or organs. However, this experiment can be laborious and time-consuming, and availability of a representative number of tissue samples is an important factor for interpretation of the results.

High-throughput gene expression analysis has allowed us to construct the equivalent of a multiple-tissue Northern blot for thousands of genes at once. We have constructed such a resource by profiling 46 human and 45 mouse tissues from diverse tissue origins. Whereas several recent studies have also described high-throughput gene expression measurements on diverse tissue sets (7–9), previous analyses of physiological gene function have been limited to identification of housekeeping genes, and clustering of genes involved in metabolic pathways and development of the central nervous system. The analysis of the data

described in the current work has a significantly different and expanded scope. Here, we use mRNA expression patterns to specifically augment gene annotation of genes with no known physiological function. Furthermore, we extend this analysis to investigate mechanisms of transcriptional regulation, to discover candidate disease markers, and to compare transcriptional profiles of gene orthologs in mouse and human. Finally, we have constructed a web resource that allows users to easily perform common queries on the data. Because these data are generated from a non-ratiometric and standardized genomic technology, expansion of this dataset in our continuing effort toward elucidating the transcriptome will easily allow inclusion of additional gene expression data from internal samples as well as those contributed by external collaborators.

Materials and Methods

Samples and Chip Hybridization. Forty-six human tissue samples and cell lines were obtained from commercial sources and previously published research collaborations, and forty-five mouse tissue samples were derived from dissections. Detailed sample descriptions can be obtained on the web site (<http://expression.gnf.org>). These samples were labeled and hybridized to either human (U95A) or mouse (U74A) high-density oligonucleotide arrays (10, 11) as described (12). Primary image analysis of the arrays was performed by using GENECHIP 3.2 (Affymetrix, Santa Clara, CA), and images were scaled to an average hybridization intensity (average difference) of 200.

Identification of Tissue-Specific Genes. For the human dataset, the set of 46 tissues, organs, and cell-lines was reduced to 25 independent and nonredundant samples (see Table 1, which is published as supporting information on the PNAS web site, www.pnas.org). All 45 mouse samples were derived from dissection and were already considered as having independent origins. Based on extensive PCR-validation of oligonucleotide array data (data not shown) and the absence/presence call provided by the GENECHIP software package, an average difference (AD) value of 200 was defined as a conservative threshold to call a gene “expressed” or present. Additionally, an AD of 200 has been estimated to represent ≈ 3 –5 copies per cell, and an expression ratio of 2-fold has previously been established as the approximate limit of sensitivity (10, 11). By using these guidelines as filtering criteria, tissue-specific genes were conservatively defined as having an AD value of greater than 200 in one tissue, and AD value of less than 100 in all other tissues.

Abbreviations: AD, average difference; GPCR, G protein-coupled receptor.

^{*}A.I.S., M.P.C., K.A.C., Y.H., J.R.W., T.W., and A.P.O. contributed equally to this work.

[†]To whom reprint requests should be addressed at: The Genomics Institute of the Novartis Research Foundation, 3115 Merryfield Row, San Diego, CA 92121-1125. E-mail: hogenesch@gnf.org.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Transcriptional Response Elements. The human dataset was filtered to select genes with expression in the pituitary gland that was 10-fold greater than median and greater than 3-fold above the median in no more than five other tissues. Thirty-four probe sets were identified that mapped to 23 unique Reference Sequence (Refseq) entries and four uncharacterized probe sets. To retrieve the promoter regions for these genes, the first 300 coding nucleotides were aligned to the human genome by using BLAST. Where significant hits (98% identity over at least 100 nucleotides) were identified, a 5-kb upstream sequence of the translational start methionine was retrieved. Because the transcriptional start sites of few genes are known, and because response elements have also been identified in the first intron of many structural genes, our searches were limited to the regions immediately 5' of the translational start methionine. By using this method, promoter regions for 18 of the 23 pituitary-enriched genes were identified. Sequences were analyzed for conserved motifs by using ALIGNACE and SCANACE [George Church, Harvard University (13)].

Prostate Cancer Profiling. Twenty-four prostate tumors and nine benign prostate tissues were profiled as described (14). To identify genes overexpressed in prostate cancer, genes were ranked by calculating the sum of three independent rank tests: the rank of [average hybridization intensity in tumor tissue (T) – average hybridization intensity in normal tissue (N)] + the rank of [average(T)/average(N)] + the rank ($-P$), where P is the P -value calculated by an unpaired, one-tailed t test. These cancer overexpressed genes were further ranked according to their average levels of expression in the gene expression atlas, with lowly expressed genes scoring highest.

Comparison of Mouse and Human Gene Expression. Putative ortholog pairs in mouse and human were identified by finding genes with common LocusLink symbols (<http://www.ncbi.nlm.nih.gov/LocusLink>). Genes that were not expressed (AD less than 200 in all tissues), and genes that were not differentially expressed (ratio of maximum expression to median expression in all tissue less than 3) were removed from the analysis. Gene expression values of the remaining 799 putative orthologs pairs were compared by Pearson's correlation coefficient.

Results and Discussion

RNA samples from 46 human and 45 mouse tissues, organs, and cell lines were hybridized to high-density gene expression arrays. To validate the data, we used PCR to amplify ORFs from cDNA libraries constructed from tissue sources where the database indicated the gene was expressed. Without any optimization of PCR conditions, this analysis resulted in the successful amplification of 82% of 1,824 targets from tissue libraries where expression was seen in the gene expression atlas (data not shown). One hundred PCR reactions were also performed in tissues where the gene expression atlas indicated no message was present, resulting in only one positive amplification (data not shown).

Examining gene expression across a panel of tissues allows us to identify both ubiquitously expressed "housekeeping genes," the focus of Warrington *et al.* (7), as well as differentially expressed genes, which we hypothesize perform specific cellular and physiological functions. In our dataset, $\approx 6.0\%$ of the interrogated genes are ubiquitously expressed, approximately the same percentage as reported in Warrington *et al.* (7.5%). Furthermore, whereas any individual tissue expresses approximately 30–40% of genes, almost all genes (90%) are expressed in at least one tissue examined. Statistical analysis (ANOVA) revealed that 78% and 82% of genes are differentially expressed in the mouse and human, respectively ($P < 0.001$). Hierarchical clustering of these differentially expressed genes shows that

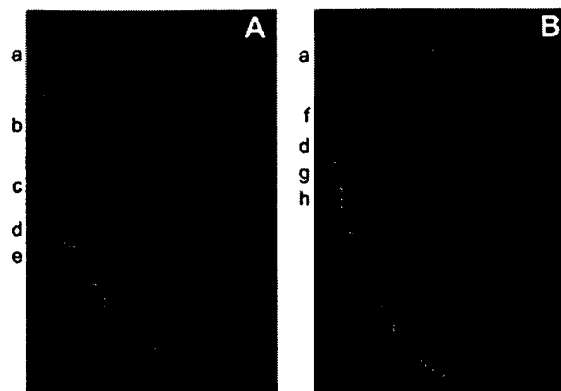
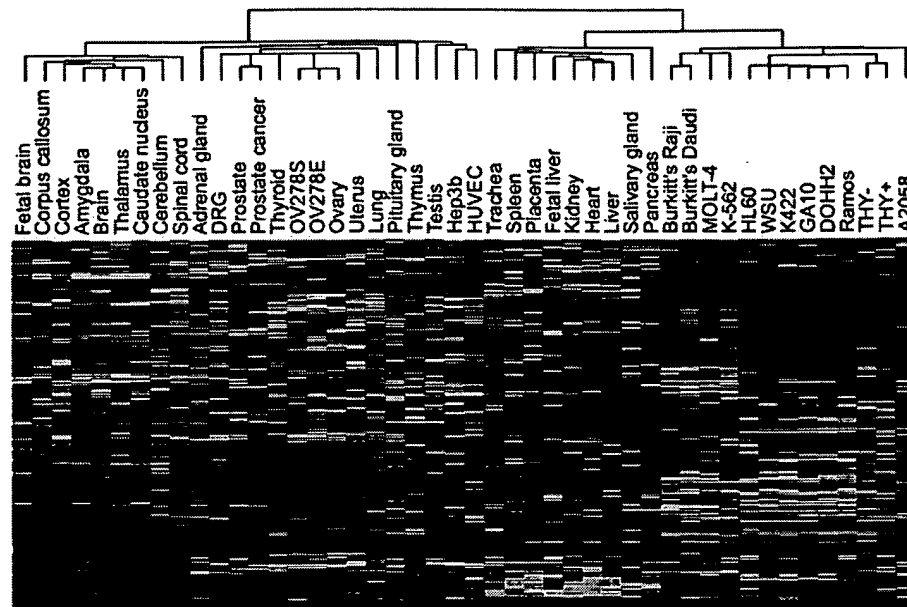


Fig. 1. Expression of tissue-specific genes. Genes with tissue-specific expression patterns were identified for all tissues in the human (A) and mouse (B) datasets. "Tissue-specific" was defined as expressed with AD greater than 200 in one tissue and less than 100 AD in all other tissues. Tissues were sorted by the number of tissue-specific genes found. The five tissues in human and mouse with the most tissue-specific genes are labeled. Replicate samples from one tissue were averaged, and genes and tissues were clustered by using CLUSTER and visualized by using TREEVIEW (25). Red, up-regulated; green, down-regulated; black, median expression. Tissue labels: a = testis, b = pancreas, c = liver, d = placenta, e = thymus, f = mammary gland, g = thyroid, and h = salivary gland).

groups of tissue-specific genes are readily identified in nearly all tissues examined. The most striking examples of these differentially regulated genes are those genes whose expression is restricted to a single tissue (Fig. 1). For example, in this dataset there are 85 human genes restricted to the testis, including several that are known to be involved in testis-function, such as SRY (sex determining region Y)-box 5 (SOX5), testicular tekton 2 (TEKT2), and zona pellucida binding protein (ZPBP). In addition, 19 genes of unknown function were identified as testis-specific, including several whose cDNAs encode large proteins (15). Similar analysis for all tissues in both mouse and human datasets identified 311 human and 155 mouse tissue-restricted genes with known function, and 76 human and 101 mouse genes whose functions were previously uncharacterized (Fig. 1; see also Tables 1 and 2, which are published as supporting information on the PNAS web site).

The integration of large-scale expression data with sequence homology-based annotation was used to obtain a more complete description of gene function. Sequence analysis of an uncharacterized protein is commonly used to identify its molecular function (e.g., kinase, protease, and transcription factor). Knowledge of the tissue expression pattern of a gene can complement this annotation by suggesting a physiological function (e.g., homeostasis, development, and proliferation) reflecting the tissues or conditions in which it is expressed. These two methods of gene annotation were integrated by mapping the tissue expression pattern of the genes represented in the database to Pfam, a database of more than 3,000 protein families and domains (6). To illustrate the utility of this approach, we used the gene expression atlas to find differentially regulated members of two large and biomedically important protein families, the G protein-coupled receptor (GPCR) and kinase families. Fig. 2 shows 312 differentially regulated members of the protein kinase family and 118 differentially regulated members of the GPCR family in the human dataset. These families include many orphan receptors and kinases of unknown function. For example, orphan receptors GPR31 and GPR9 showed enriched expression in the pancreas, suggesting a role for these proteins in digestion or hormone secretion. Specific expression patterns of proteins can

Kinases (312 genes)



GPCRs (118 genes)

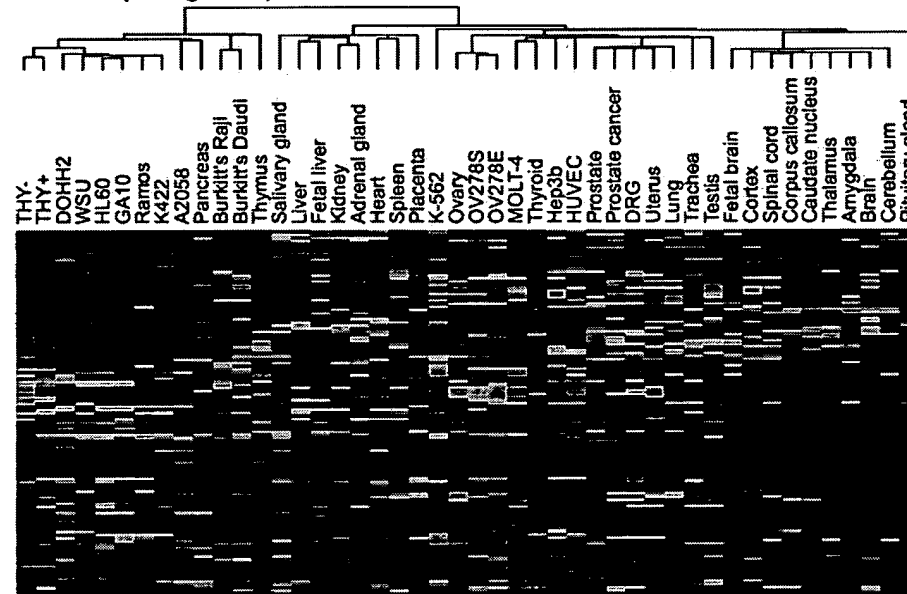


Fig. 2. Differential expression of GPCRs and kinases. Pfam was used to identify GPCRs (PF00001, PF00002, and PF00003) and kinases (PF00069, PF00433, PF00454, and PF00625) from the genes interrogated in the gene expression atlas. Data were filtered to remove genes that were not expressed in the atlas (max AD < 200) and not differentially expressed (ANOVA $P > 0.05$), and the remaining genes were visualized as described previously. The gene identities for these Pfam families, as well as for all Pfam families, can be viewed on the web site (<http://expression.gnf.org>).

also be a criterion for selecting therapeutic targets, because the primary effect of modulating their function will likely be restricted to their target tissue. We also used the gene expression atlas to identify candidate protein–protein interaction and enzyme–substrate pairs. For example, we used the gene expression atlas to find a testis-specific GPCR kinase, GPRK2L (16), and fifteen GPCRs that are detectably expressed in testis. We suggest

that these GPCRs represent the most likely substrate candidates for GPRK2L. This approach may be generally useful for decoding physiologically relevant biochemical interactions.

Together with the recent availability of the human genome sequence, coexpressed clusters of genes were used to investigate mechanisms of transcriptional regulation. To illustrate this approach, we identified genes whose expression was enriched in the

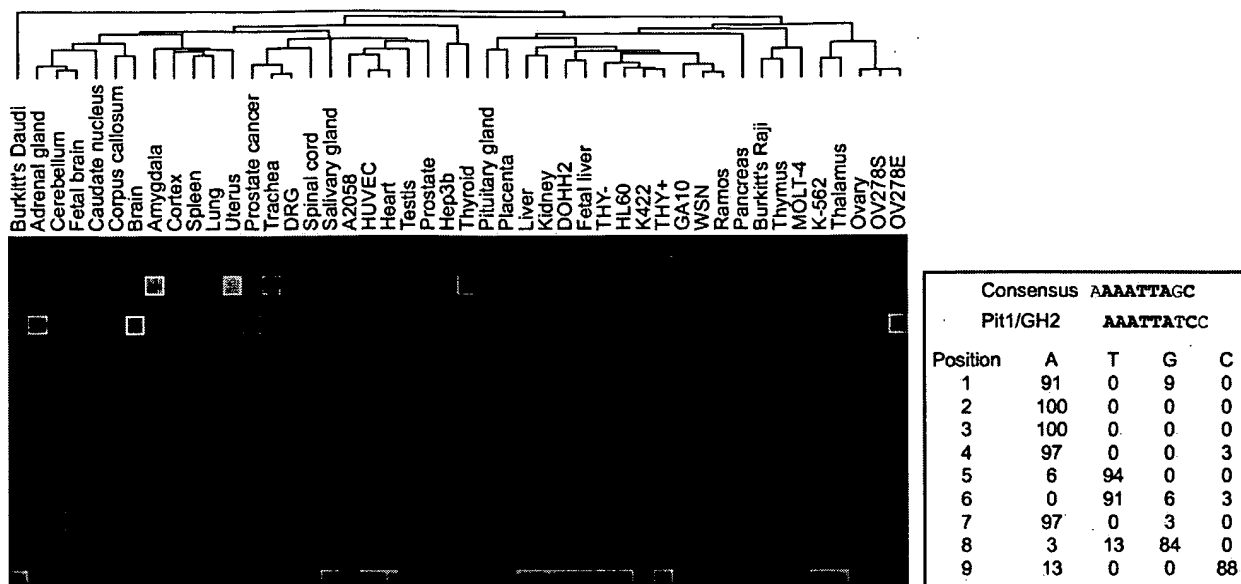


Fig. 3. Identification of pituitary-specific response elements. The gene expression atlas was used to identify pituitary-enriched genes (Left). Genomic sequence up to 5 kb upstream of the translational start methionine was searched for conserved motifs. On the Right is a potential regulatory element identified in the upstream genomic sequence of the genes in this cluster. This element is similar to a previously described Pit1 binding site from the growth hormone 2 structural gene.

pituitary gland, a tissue where specific regulation has been previously characterized (17). Twenty-three unique genes were identified, including known growth factors and peptide hormones. Four transcription factors were included in this list, two of which, Pit1 and Pitx2, were previously implicated in the regulation of pituitary-specific gene expression (17). Of these 23 genes, we were able to retrieve 18 promoter regions from the human genome assembly. To identify potential regulatory elements, we used an unbiased word-based methodology previously used in the study of prokaryotes, viruses, yeast, and *Arabidopsis* (13, 18, 19) to search the promoter regions of these genes for conserved motifs. This process identified a site highly similar to the Pit1 recognition site from the growth hormone 1 promoter that is conserved in 14 of these 18 genes (Fig. 3; ref. 20). Some of these have been previously identified as targets of Pit1, including prolactin, thyroid-stimulating hormone, the glycoprotein α subunit, and Pit1 itself. Several of these genes were unknown as potential targets of Pit1, demonstrating that the general approach of pairing tissue-specific response elements with tissue-restricted transcription factors is likely to yield novel insights into the mechanisms of complex transcriptional regulation.

This gene expression atlas was also used to identify potential markers for human disease by comparing transcriptional profiles of pathological samples to the normal transcriptome. Genes with disease-restricted expression are highly desirable both as markers and as pharmacologic targets, because selective expression imparts the specificity required for successful disease-specific targeting approaches [e.g., BCR-ABL and STI571 (21)]. In this study, we identified genes specifically up-regulated in prostate cancer samples that were lowly expressed or absent in other tissues in the database. Proof-of-concept was provided by the identification of several known prostate- and prostate cancer-specific genes including prostate-specific membrane antigen (PSMA), human kallikrein 2 (hK2), and the recently described transmembrane serine protease 2 (TMPRSS2), which although expressed in other body tissues, is most notably expressed in the

prostate (Fig. 4; ref. 22). We also discovered genes whose up-regulated expression in prostate carcinoma has not yet been previously described, including the human homologs of the *Drosophila* transcription factor single-minded, SIM2, and the lady bird late gene, LBX1. In addition, several genes with completely uncharacterized function were identified that are being pursued as potential novel cancer-specific genes. Interrogation of gene expression profiles derived from cancer and other pathological conditions in the context of normal body tissues is likely to return a battery of genes important in understanding disease mechanism and diagnoses. Furthermore, those genes that fall into protein families amenable to pharmacologic perturbation may provide entry points for the design of novel and specific therapeutics.

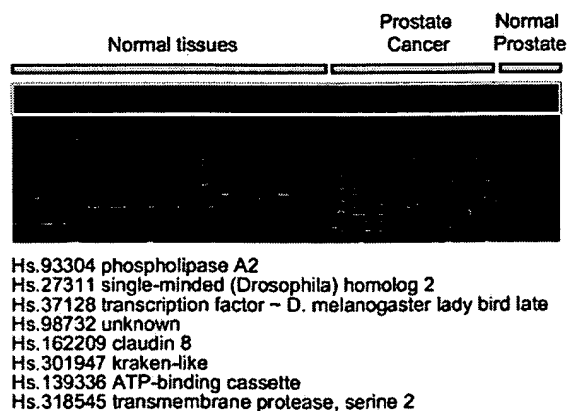


Fig. 4. Potential markers for prostate cancer were identified by comparing gene expression in normal tissues with normal and tumor prostate samples. Fifty candidate makers are visualized here, and the top eight gene identities are shown.



organisms (<http://www.ncbi.nlm.nih.gov/HomoloGene>). We would predict that true orthologs would have conserved patterns of mRNA expression reflecting the common physiological function of a gene in mice and humans. Conversely, genes of divergent function may demonstrate protein sequence and mRNA expression divergence between the two species. A set of

putative orthologs was identified by searching for mouse and human genes with a common LocusLink symbol, and further restricted for this analysis to genes that showed detectable and differential expression. The expression patterns of these 799 putative ortholog pairs were compared across the 16 tissues in common between our mouse and human datasets. This analysis revealed that half of all mouse and human orthologs have correlation in their expression patterns of 0.6 or better (Fig. 5A). Visualization of these highly correlated transcripts revealed striking similarity in the patterns of gene expression between mice and human (Fig. 5B). Conversely, there were also many examples of low and even negative correlation of expression between the two species. For example, the human extracellular matrix protein collagen XV is most highly expressed in placenta, whereas in mice the putative ortholog is most highly expressed in the uterus (Fig. 5C). Primary sequence comparisons of the mouse and human collagen XV genes revealed that the mouse harbors seven collagenous domains to nine for the human gene (23). In addition, although the conserved C-terminal endostatin domain predicts a role in angiogenesis, inactivation of the mouse structural gene by homologous recombination revealed a normal vasculature (24). Taken in sum, these data support the hypothesis that the physiological role of collagen XV is different

between the two species. Thus, expression analysis can supplement primary amino acid sequence homology in ascertaining whether a gene has conserved function between a model organism and the organism it seeks to model.

In conclusion, this significant fraction of the human and mouse transcriptome provides a powerful approach to analyze gene function. The extension of this database with additional samples and more comprehensive gene expression arrays will further increase its utility. We have also created a free and publicly accessible web site (<http://expression.gnf.org>) that allows researchers to query the mouse and human datasets based on gene name, keyword, protein family, or accession number. Users may also query the data by expression pattern to identify genes present in any tissue or combination of tissues represented in the database. It is our hope that this freely available public resource will enable researchers worldwide to exploit the emerging transcriptome to further biomedical research.

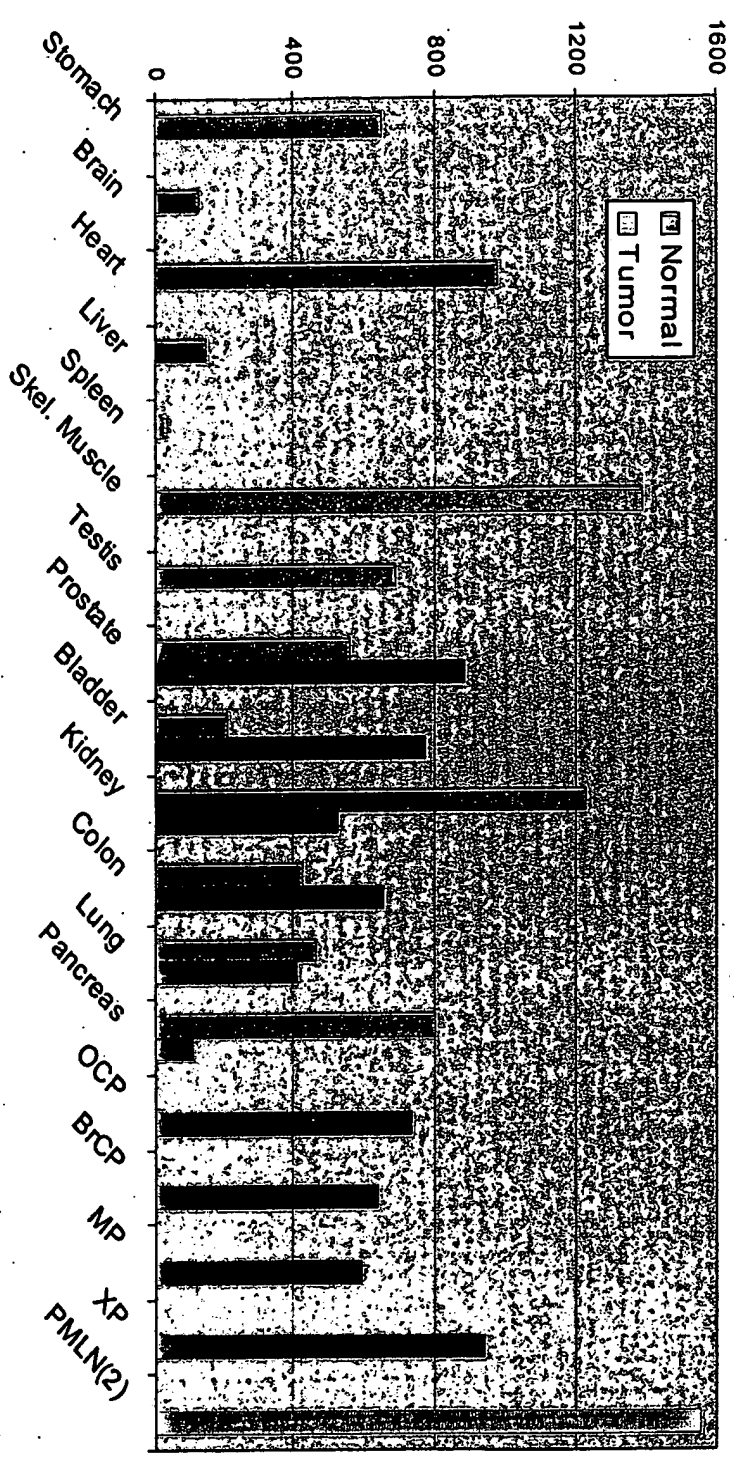
We thank David Lockhart and Lisa Wodicka for helpful discussions, and Jennifer Villaseñor for excellent technical assistance. We also thank Cheng Li and Wing Hung Wong for statistical advice, and Martha Bulyk for helpful comments and suggestions. A.I.S. acknowledges the Achievement Rewards for College Scientists (ARCS) Foundation of San Diego and the La Jolla Interfaces in Science Program for predoctoral support.

- Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., et al. (2001) *Nature (London)* 409, 860–921.
- Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., et al. (2001) *Science* 291, 1304–1351.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* 215, 403–410.
- Eddy, S. R., Mitchison, G. & Durbin, R. (1995) *J. Comput. Biol.* 2, 9–23.
- Burks, C., Fickett, J. W., Goad, W. B., Kanchisa, M., Lewitter, F. I., Rindone, W. P., Swindell, C. D., Tung, C. S. & Bilosky, H. S. (1985) *Comput. Appl. Biosci.* 1, 225–233.
- Sonnhammer, E. L., Eddy, S. R. & Durbin, R. (1997) *Proteins* 28, 405–420.
- Warrington, J. A., Nair, A., Mahadevappa, M. & Tsyganskaya, M. (2000) *Physiol. Genomics* 2, 143–147.
- Miki, R., Kadota, K., Bono, H., Mizuno, Y., Tomaru, Y., Carninci, P., Itôh, M., Shibata, K., Kawai, J., Konno, H., et al. (2001) *Proc. Natl. Acad. Sci. USA* 98, 2199–2204.
- Penn, S. G., Rank, D. R., Hanzel, D. K. & Barker, D. L. (2000) *Nat. Genet.* 26, 315–318.
- Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., Gallo, M. V., Chee, M. S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H. & Brown, E. L. (1996) *Nat. Biotechnol.* 14, 1675–1680.
- Wodicka, L., Dong, H., Mittmann, M., Ho, M. H. & Lockhart, D. J. (1997) *Nat. Biotechnol.* 15, 1359–1367.
- Sandberg, R., Yasuda, R., Pankratz, D. G., Carter, T. A., Del Rio, J. A., Wodicka, L., Mayford, M., Lockhart, D. J. & Barlow, C. (2000) *Proc. Natl. Acad. Sci. USA* 97, 11038–11043.
- Hughes, J. D., Estep, P. W., Tavazoie, S. & Church, G. M. (2000) *J. Mol. Biol.* 296, 1205–1214.
- Welsh, J. B., Sapinoso, L. M., Su, A. I., Kern, S. G., Wang-Rodriguez, J., Moskaluk, C. A., Frierson, H. F., Jr., & Hampton, G. M. (2001) *Cancer Res.* 61, 5974–5978.
- Nagase, T., Ishikawa, K., Suyama, M., Kikuno, R., Miyajima, N., Tanaka, A., Kotani, H., Nomura, N. & Ohara, O. (1998) *DNA Res.* 5, 277–286.
- Sallese, M., Mariggio, S., Collodel, G., Moretti, E., Piomboni, P., Baccetti, B. & De Blasi, A. (1997) *J. Biol. Chem.* 272, 10188–10195.
- Rosenfeld, M. G., Briata, P., Dasen, J., Gleiberman, A. S., Kiousi, C., Lin, C., O'Connell, S. M., Ryan, A., Szeto, D. P. & Treier, M. (2000) *Recent Prog. Horm. Res.* 55, 1–13.
- McGuire, A. M., Hughes, J. D. & Church, G. M. (2000) *Genome Res.* 10, 744–757.
- Harmer, S. L., Hogenesch, J. B., Straume, M., Chang, H. S., Han, B., Zhu, T., Wang, X., Kreps, J. A. & Kay, S. A. (2000) *Science* 290, 2110–2113.
- Scully, K. M., Jacobson, E. M., Jepsen, K., Lunyak, V., Viadiu, H., Carriere, C., Rose, D. W., Hooshmand, F., Aggarwal, A. K. & Rosenfeld, M. G. (2000) *Science* 290, 1127–1131.
- Mauro, M. J. & Druker, B. J. (2001) *Curr. Opin. Oncol.* 13, 3–7.
- Lin, B., Ferguson, C., White, J. T., Wang, S., Vessella, R., True, L. D., Hood, L. & Nelson, P. S. (1999) *Cancer Res.* 59, 4180–4184.
- Eklund, L., Muona, A., Lietard, J. & Pihlajaniemi, T. (2000) *Matrix Biol.* 19, 489–500.
- Eklund, L., Pihola, J., Komulainen, J., Sormunen, R., Ongvarrasopone, C., Fassler, R., Muona, A., Ilves, M., Ruskoaho, H., Takala, T. E. & Pihlajaniemi, T. (2001) *Proc. Natl. Acad. Sci. USA* 98, 1194–1199.
- Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. (1998) *Proc. Natl. Acad. Sci. USA* 95, 14863–14868.

BEST AVAILABLE COPY

RNA Expression of HER2/neu in Human Normal Tissues

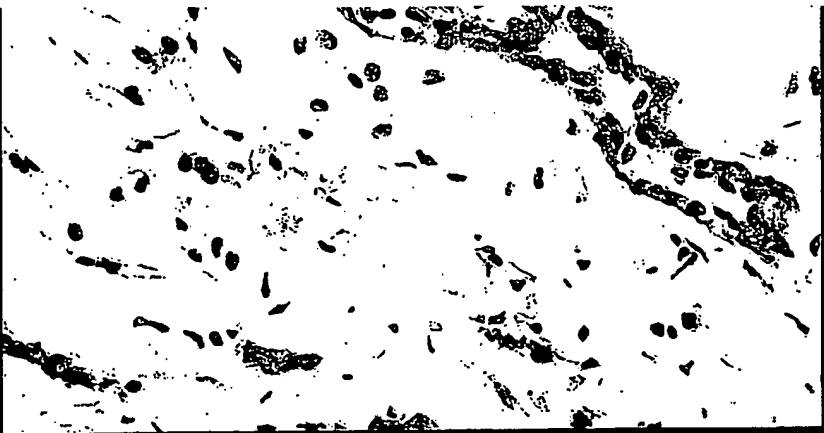
Her2/neu



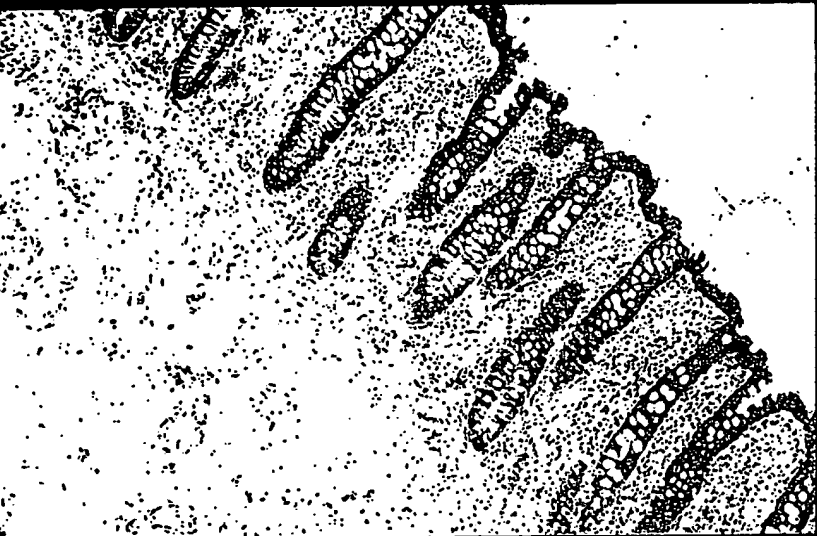
BEST AVAILABLE COPY

HER2/neu Protein Expression in Human Tissues

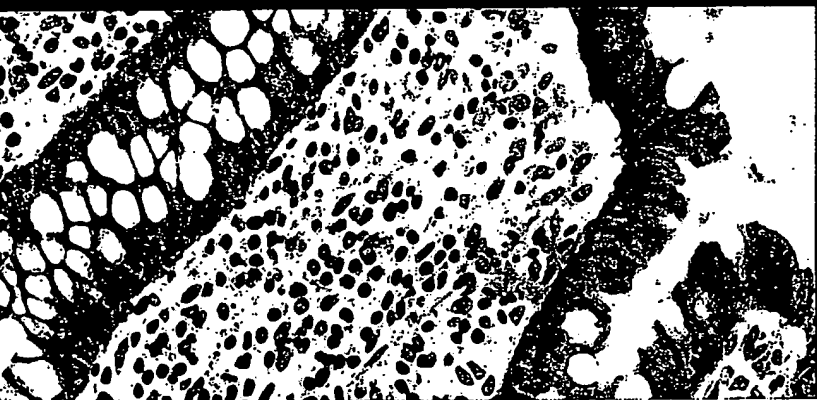
Kidney



Colon



Colon



Gene amplification and overexpression of *HER2* in renal cell carcinoma

Z. LATIF, A.D. WATTERS, J.M.S. BARTLETT, M.A. UNDERWOOD and M. AITCHISON*

Department of Surgery, Glasgow Royal Infirmary, and *Department of Urology, Gartnavel General Hospital, Glasgow, UK

Objective To determine the frequency of *HER2* genetic abnormalities in renal cell carcinoma (RCC) and hence assess the potential suitability of Herceptin™ immunotherapy.

Patients and methods Tumours from 27 patients with RCC were assessed; all patients had undergone nephrectomy. Benign renal tissue from the nephrectomy specimens was studied in seven patients. Gene amplification was assessed using fluorescent *in-situ* hybridization, and protein over-expression using immunohistochemistry.

Results Twenty-four patients had clear cell renal carcinoma, two had papillary renal carcinoma and one a sarcomatoid variant carcinoma. There was no

HER2 amplification in the tumours or the benign renal tissue. Polysomy 17 was detected in 11 of 27 tumours (41%) and increased *HER2* copy number in seven (26%). Both polysomy 17 and increased *HER2* copy number were absent in the benign renal tissue. Three tumours (11%) and six of the seven benign renal tissue samples over-expressed the *HER2* protein.

Conclusions *HER2* amplification is absent and protein over-expression uncommon in RCC. This casts doubt on the suitability of Herceptin™ in the treatment of RCC.

Keywords renal carcinoma, *HER2*, Herceptin™, FISH, immunohistochemistry

Introduction

Patients with locally advanced or metastatic RCC have a poor prognosis; the 5-year survival for patients with stage IV disease is 10% [1], reflecting that RCC is largely resistant to conventional therapeutic regimens [2]. Immunotherapy using interleukin-2 and interferon- α combined has shown response rates of up to 18.6% in patients with metastatic disease, but is associated with significant side-effects [3]. Negrier *et al.* [3] reported grade 3 and 4 adverse events like hypotension (resistant to vasopressor agents) and fever in 45% and 35% of patients, respectively. A better understanding of the molecular genetics of RCC may help in the development of new therapeutic agents. Aneuploidy of chromosome 17 has been reported in papillary RCC [4,5]. The *HER2/c-erbB2* gene is located on chromosome 17 and encodes a transmembrane type I tyrosine kinase growth factor receptor with 50% homology with the EGFR [6-8]. Over-expression of *HER2* has been reported in breast [9-11], ovarian [9], lung [12] and salivary gland tumours [13], and is associated with a poorer prognosis. In a recent review examining the results of 47 studies involving >22 600 patients with breast cancer, *HER2*-positive status was an independent predictor of poor prognosis in multivariate analysis [14]. Herceptin™

(*rhu-mAb-HER2*, Trastuzumab, Genentech Inc., San Francisco, CA) is the humanized equivalent of the murine 4D5 mAb and is targeted against the *HER2* cell-surface receptor. Phase II and phase III clinical trials in patients with breast cancer over-expressing the *HER2* oncogene showed response rates of 11-15% [15,16]. A recent study showed that 40% of RCCs over-express the *HER2* protein and 17% show gene amplification [17,18]. However, other studies have not confirmed these findings, suggesting that the *HER2* expression and amplification rates are lower [19-21]. These differences may be partly explained by the different laboratory techniques used in the various studies. Determination of protein over-expression by immunohistochemistry (IHC) is highly dependent on the antibody selected and the methods used. Fluorescent *in-situ* hybridization (FISH) is the approved diagnostic method for determining gene amplification, allowing *in-situ* analysis of gene copy number. The Glasgow Royal Infirmary is one of three UK centres evaluating *HER2* status in breast cancer diagnosis [22]. In the present study we evaluated the *HER2* status of 27 RCCs and seven benign renal tissues using FISH and IHC.

Patients and methods

Tumours from patients with a pathological diagnosis of RCC and attending the Gartnavel General Hospital

Accepted for publication 2 September 2001

between 1997 and 1999 were included in the study. All patients underwent nephrectomy and tumours were assigned TNM [23] and Robson stages [24] and grades [25]. Benign (normal) renal tissue was identified from the RCC nephrectomy specimens. IHC was performed using the CB11 mouse mAb and the Nexus II automated staining system (both from Ventana, UK). Formalin-fixed paraffin-embedded 5 µm sections and positive/negative controls were used in each run. Antigens were retrieved in a pressure cooker for 12 min (1 L distilled water and 1.8 mmol of EDTA at pH 8 in an 850-W microwave oven), followed by a 20-min cooling period. Endogenous biotin was blocked by applying avidin for 15 min and then biotin for 15 min (SP-2001, Vector Laboratories, Burlingame, USA). The slides were transferred to the Nexus II and the basic diaminobenzidine detection kit (Ventana) applied; 100 µL of CB11 was added manually to each slide as appropriate and incubated in the Nexus II for 32 min. The result was scored by two independent observers. Routine sections stained with haematoxylin and eosin were examined to identify areas of tumour or benign renal tissue, and corresponding areas marked on the IHC sections. IHC was scored according to both the intensity and number of cells stained. No staining or membrane staining in <10% of cells was scored as 0; weak staining of the membrane in >10% of cells was scored as 1+ moderate membrane staining in >10% of cells was scored as 2+, and strong membrane staining in >10% of cells as 3+. Cytoplasmic staining was excluded. A score of 2+ or 3+ was regarded as 'positive' and a score of 0 or 1+ as 'negative' [26].

FISH was performed as previously described [27,28]; briefly, slides were pretreated with 0.2 mol/L HCl, 8% sodium thiosulphate at 80°C and 0.5% pepsin at 37°C. Sections were post-fixed in 10% neutral buffered formalin. The extent of digestion was estimated by applying 0.5 µg/mL 4,6-diamidino-2 phenylindole-2 hydrochloride in Vectashield (Vectorlabs, Peterborough, UK) antifade mounting medium. Sections were viewed at ×400 using a microscope. The target DNA was denatured with 100 µL of 70% formamide in 2 × saline/citrate buffer, 10 µL of the probe applied and the slides incubated overnight in an Omnislide at 37°C (Hybaid, UK). Chromosome 17 and HER-2 copy number was evaluated using a direct dual-labelled technique, with the Pathvysion™ HER-2 DNA probe kit (Vysis, UK). This incorporated a Spectrum Green-labelled α-satellite probe for chromosome 17 and a Spectrum Orange-labelled HER2 probe. Again, serial sections stained with haematoxylin and eosin were first examined microscopically to locate tumour areas and benign renal tissue. FISH-stained sections were then scanned at ×400 to locate these regions. Three areas were identified and 20 nuclei assessed per area by two independent observers

with ×40 or ×100 objectives. Signals per nucleus for chromosome 17 (green) and HER2 (orange) were counted and the results recorded manually; in all, 60 nuclei were assessed. Gene amplification was defined as a HER2/chromosome 17 ratio of >2. Based on values from controls tissues in our laboratory, the normal values for chromosome 17 were 1.35–1.85 (1.61 ± 3 sd) and for HER2 were 1.51–1.99 (1.75 ± 3 sd). Values of >3 sd over the mean were defined as polysomic (for chromosome 17) or showing increased HER2 copy number.

Results

Twenty-seven tumours were assessed from 19 men and eight women; 24 tumours were of the clear cell type, two were papillary and one was of a spindle-cell variant. The clinical details of the 27 patients are summarized in Table 1, based on the TNM classification and more generally by the Robson classification (Table 2).

Table 1 Summary of the clinical details, FISH and IHC results of the 27 patients with RCC

Case/Sex	Grade	TNM	Copy number		
			Chrom 17	HER2	IHC
1/M	2	T3N0M0	1.51	1.68	0
2/F	2	T2N0M0	1.78	1.71	0
3/M*	4	T3N2M0	1.59	1.55	0
4/M	2	T1N0M0	1.85	1.69	0
5/M*	3	T3N0M0	2.66	2.88	0
6/F	3	T1N0M0	1.73	1.69	0
7/F	3	T2N0M0	2.5	2.88	0
8/F	4	T1N0M0	1.2	1.21	0
9/F	3	T3N0M0	1.73	1.74	2
10/M	2	T3N0M0	1.87	1.79	0
11/M	2	T2N0M0	1.19	1.18	0
12/M	3	T1N0M0	2.8	3.02	0
13/F	1	T3N0M0	1.71	1.63	0
14/M	3	T3NXM0	2.41	2.48	0
15/M	3	T3N1M0	1.9	1.78	0
16/M	3	T1N0M0	1.75	1.78	0
17/M	3	T2NXM0	1.58	1.69	0
18/M†	4	T4N0M0	2.03	1.65	0
19/M	3	T2N0M0	1.7	1.65	0
20/M	4	T3N0M0	2.16	2.2	0
21/M	2	T2N0M0	2.53	2.92	2
22/M	3	T1N0M0	1.74	1.87	2
23/M	2	T1NXM0	1.73	1.84	0
24/M	2	T3N0M0	2.32	2.37	0
25/M	3	T3N0M0	1.88	1.85	0
26/F	3	T2NXM0	1.83	1.71	1
27/F	3	T2NXM0	1.75	1.78	0

*Represents the two papillary tumours and †the spindle cell carcinoma.

Table 2 The distribution of stage and tumour type in the tumours in the study, and the distribution of the RCCs with polysomy 17 and increased HER-2 copy number; both these genetic events were more frequent in more advanced stages

Tumour type or result	Stage				N
	I	II	III	IV	
Clear cell	6	6	7	5	24
Papillary	0	0	1	1	2
Spindle cell variant	0	0	0	1	1
Total	6	6	8	7	27
Result:					
Polysomy 17	0	3	4	4	
Increased HER2 copy number	0	2	2	3	
IHC (2+ or 3+)	2	1	0	0	

All FISH hybridizations were successful; there was no gene amplification in any of the tumours or the benign renal tissue. The median (range) HER2/chromosome 17 number was 0.99 (0.8–1.17) for the tumours and 0.99 (0.93–1.15) for benign tissue. The median chromosome 17 copy number for the tumours was 1.78 (1.19–2.8) and 11 (41%) were polysomic for chromosome 17. The median chromosome 17 copy number for benign renal tissue was 1.59 (1.5–1.79) and hence none was polysomic for chromosome 17. The median HER2 copy number for the tumours was 1.76 (1.18–3.02) and hence seven (26%) had increased HER2 copy number (all seven were also polysomic for chromosome 17). The median HER2 copy number for benign renal tissue was 1.68 (1.4–1.8) and hence none had increased HER2 copy number.

All the IHC was completed successfully; three tumours (11%) over-expressed the HER2 protein (2+); all other tumours were negative. Six of the seven benign renal tissues over-expressed the HER2 protein (3+); all these positive samples had a particular distribution of positively staining cells, confined to the distal nephron (Fig. 1a,b).

Discussion

Patients with metastatic RCC have a 5-year survival of only 10% and RCC is resistant to conventional chemotherapeutic agents [1,2]. The study of the molecular genetics of RCC may help to develop new therapeutic agents. Alterations of genes that function as regulators of cell growth and differentiation are considered to be crucial in the progression of human cancers [29]. Numerous genetic aberrations have been documented in RCC and many are associated with a poor clinical outcome [30]. For example, the EGFR (on chromosome 7) is over-expressed in 80% and there is

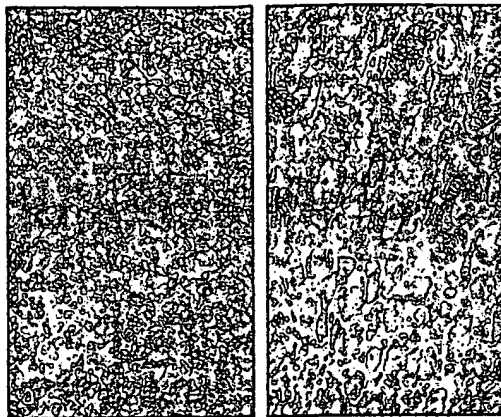


Fig. 1. Positively staining: a, RCC tissue, scored as a '2+' and b, benign renal tissue; note the particular distribution of positively staining cells confined to the distal nephron in the latter. Both $\times 160$.

polysomy of chromosome 7 in 75% of RCCs, predicting a poor prognosis [31,32]. Polysomy 7 is linked to polysomy 17 in RCC [4,5] and the HER2 oncogene is on chromosome 17. Over-expression/gene amplification of HER2 has been reported for many human tumours and represents a poor prognostic factor in several [9,11–13]. HER2 gene amplification and protein over-expression are thought to be important in oncogenic transformation, tumorigenesis and metastasis [33]. For example, in breast cancer, patients whose tumours have gene amplification/protein over-expression (15–30% of all patients) have a poorer prognosis. These patients respond to clinical treatment with the mAb Herceptin, which is targeted against the HER2 cell surface receptor [15,16]. Herceptin has cytostatic growth inhibitory effects against breast cancer cells, and induces antibody-dependent cellular cytotoxicity against human tumour cell lines, contributing to its antitumour activity [33].

There is a wide variation in the reported results for HER2 assessment in RCC, partly because of the differing laboratory techniques applied in the various studies. Rotter *et al.* [18] used slot-blot analysis and found no gene amplification in any of 24 tumours. Similarly Weldner *et al.* [19] (six tumours), Freeman *et al.* [20] (13 tumours) and Stuum *et al.* [21] (34 tumours) showed no gene amplification by Southern blot analysis in RCCs. Classical techniques for detecting gene amplification, like Southern blotting, need a large quantity of DNA and all have the problem of varying tumour content within a given specimen, which renders the results unreliable if the percentage of tumours cells is low in a sample [10]. More recently Zhang *et al.* [17] (70 tumours) used differential PCR and reported that 17% of tumours showed gene amplification for HER2 in addition

to a significant association with tumour stage. The conclusion was that HER2 abnormalities may be involved in the pathogenesis of RCC. Differential PCR must be rigorously controlled if it is to be used successfully to quantify mRNA or gene copy number [34]. Furthermore, the use of tissue homogenates, as used for southern analyses (see above), may further compromise the sensitivity of this technique. FISH is the preferred technique for visualizing HER2 in breast cancer diagnostics, because it assesses DNA in individual cells using a specific fluorescently labelled probe. Hence there is no need to microdissect tumour cells or to isolate nucleic acids before labelling, as with Southern blotting, and indeed FISH has been shown to be superior to other techniques in detecting HER2 amplification on formalin-fixed, paraffin-embedded material [10]. Comparing the results of studies estimating protein over-expression of HER2 is difficult because all have used different antibodies and scoring criteria. However, Stuurm *et al.* [21] and Zhang *et al.* [17] reported that 64% and 40% of RCCs over-expressed the HER2 protein using IHC, and that HER2 was important in the progression of RCC. In contrast, Rotter *et al.* [18] reported that HER2 protein over-expression was uncommon in RCC and that HER2 was not important in the progression of RCC. Consistent with this conclusion, others [19,20] reported that mRNA levels were lower in RCCs than in normal tissue. Hence the exact role of HER2 in the pathogenesis of RCC remains unclear.

In the present study of 27 RCCs and seven benign renal tissues examined for HER2 abnormalities using FISH and IHC, there was no gene amplification in any of the tissues. However, polysomy 17 occurred in 41% and increased HER2 copy number in 26% of the tumours. Furthermore, polysomy 17 and increased HER2 copy number were more frequent in stage III and stage IV cancers (Table 2). Both polysomy 17 and increased HER2 copy number were absent in the benign renal tissue.

Protein was over-expressed in 11% of the tumours at 2+ and in six of seven of the benign renal tissues at 3+. Hence protein was strongly over-expressed (3+) only in the benign renal tissue, confined to the distal parts of the nephron. There was no staining in the proximal tubules, which are the cells thought to give rise to RCC. This is consistent with results reported previously [19,20,35], suggesting that under-expression of HER2 mRNA is characteristic of RCCs. Consistent with this, Rotter *et al.* [18] suggested that reduced HER2 protein and gene expression were inversely related to tumour differentiation, with less well differentiated tumour cells having lower HER2 expression. Current evidence suggests that in breast cancer only patients with tumours that are 3+ on IHC or 2+ with gene

amplification, as shown by FISH, respond to Herceptin immunotherapy [36], implying that the advanced RCCs in the current study may be unresponsive to Herceptin.

The present results suggest that gene amplification and strong protein over-expression of HER2 is absent in RCC. This casts doubt on the suitability of Herceptin immunotherapy in the management of RCC.

Acknowledgements

The authors thank Professor T.G. Cooke for his support. This research was funded by The British Urology Foundation (BUF/Pfizer Scholarship 2000/2001) and The Royal College of Surgeons of Edinburgh.

References

- 1 Selli C, Hinshaw WM, Woodard BH, Paulson DF. Stratification of risk factors in renal cell carcinoma. *Cancer* 1983; 52: 899-903
- 2 Yagoda A, Abi-Rached B, Petrylak D. Chemotherapy for advanced renal-cell carcinoma: 1983-93. *Semin Oncol* 1995; 22: 42-60
- 3 Negrier S, Escudier B, Lasset C *et al.* Recombinant human interleukin-2, recombinant human interferon alfa-2a, or both in metastatic renal cell carcinoma. *New Engl J Med* 1998; 338: 1272-8
- 4 Corless CL, Aburatani H, Fletcher JA, Houseman DE, Ami MB, Weinberg DS. Papillary renal cell carcinoma. Quantitation of chromosomes 7 and 17 by FISH, analysis of chromosome 3p for LOH, and DNA ploidy. *Diag Mol Pathol* 1996; 5: 53-64
- 5 Brown JA, Borell TJ, Qian J, Bostwick DG, Jenkins RB. Simultaneous chromosome 7 and 17 gain and sex chromosome loss provide evidence that metanephric adenoma is related to papillary renal cell carcinoma. *J Urol* 1997; 158: 370-4
- 6 Schechter AL, Hung MC, Vaidyanathan L *et al.* The neu gene. An erbB-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. *Science* 1985; 229: 976-8
- 7 Bargmann CI, Hung MC, Weinberg RA. The neu oncogene encodes An epidermal growth factor receptor-related protein. *Nature* 1986; 319: 226-30
- 8 Akiyama T, Sudo C, Ogawara H, Toyoshima K, Yamamoto T. The product of the human c-erbB-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science* 1986; 232: 1644-6
- 9 Slamon DJ, Godolphin W, Jones LA *et al.* Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 1989; 244: 707-12
- 10 Pauletti G, Godolphin W, Press MF, Slamon DJ. Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence *in situ* hybridization. *Oncogene* 1996; 13: 63-72

- 11 Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer. Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987; 235: 177-82
- 12 Schneider PM, Hung MC, Chiocca SM *et al*. Differential expression of the c-erbB-2 gene in human small cell and non-small cell lung cancer. *Cancer Res* 1989; 49: 4968-71
- 13 Press MF, Pike MC, Hung G *et al*. Amplification and overexpression of HER-2/neu in carcinomas of the salivary gland: correlation with poor prognosis. *Cancer Res* 1994; 54: 5675-82
- 14 Revillion F, Bonnetterre J, Peyrat JP. ERBB2 oncogene in human breast cancer and its clinical significance. *Eur J Can* 1998; 34: 791-808
- 15 Cobleigh MA, Vogel CL, Tripathy D *et al*. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999; 17: 2639-48
- 16 Baselga J, Tripathy D, Mendelsohn J *et al*. Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/neu-overexpressing metastatic breast cancer. *J Clin Oncol* 1996; 14: 737-44
- 17 Zhang XH, Takenaka I, Sato C, Sakamoto H. p53 and HER-2 alterations in renal cell carcinoma. *Urology* 1997; 50: 636-42
- 18 Rotter M, Block T, Busch R, Thanner S, Hofler H. Expression of HER-2/neu in renal-cell carcinoma. Correlation with histologic subtypes and differentiation. *Int J Cancer* 1992; 52: 213-7
- 19 Weidner U, Peter S, Strohmeier T, Hussnatter R, Ackermann R, Sies H. Inverse relationship of epidermal growth factor receptor and HER2/neu gene expression in human renal cell carcinoma. *Cancer Res* 1990; 50: 4504-9
- 20 Freeman MR, Washecka R, Chung LW. Aberrant expression of epidermal growth factor receptor and HER-2 (erbB-2) messenger RNAs in human renal cancers. *Cancer Res* 1989; 49: 6221-5
- 21 Stumm G, Eberwein S, Rostock-Wolf S *et al*. Concomitant overexpression of the EGFR and erbB-2 genes in renal cell carcinoma (RCC) is correlated with dedifferentiation and metastasis. *Int J Cancer* 1996; 69: 17-22
- 22 Ellis IO, Dowsett M, Bartlett J *et al*. Recommendations for HER-2 testing in the UK. *J Clin Pathol* 2000; 53: 890-2
- 23 Hermanek P, Hutter RVP, Sobin LH, Wagner G, Wittekind Ch. TNM Atlas. 4th edn. International Union against Cancer, 1997, 296-302
- 24 Robson CJ, Churchill BM, Anderson W. The results of radical nephrectomy for renal cell carcinoma. *J Urol* 1969; 101: 297-301
- 25 Fuhrman SA, Lasky LC, Limas C. Prognostic significance of morphologic parameters in renal cell carcinoma. *Am J Surg Pathol* 1982; 6: 655-63
- 26 Van de Vijver MJ. Assessment of the need and appropriate method for testing for the human epidermal growth factor receptor-2 (HER2). *Eur J Can* 2001; 37: S11-7
- 27 Bartlett JMS, Watters AD, Ballantyne SA, Going JJ, Grigor KM, Cooke TG. Is chromosome 9 loss a marker of disease recurrence in transitional cell carcinoma of the urinary bladder? *Br J Cancer* 1998; 77: 2193-8
- 28 Watters AD, Ballantyne SA, Going JJ, Grigor KM, Bartlett JMS. Aneuploidy of chromosomes 7 and 17 predicts the recurrence of transitional cell carcinoma of the urinary bladder. *BJU Int* 2000; 85: 42-7
- 29 Strohmeier GT, Slamon DJ. Proto-oncogenes and tumour suppressor genes in human urological malignancies. *J Urol* 1994; 151: 1479-97
- 30 Moch H, Presti JC, Sauter G *et al*. Genetic aberrations detected by comparative genomic hybridisation are associated with clinical outcome in renal cell carcinoma. *Cancer Res* 1996; 56: 27-30
- 31 Moch H, Sauter G, Bucholz N, Waldman FM, Mihatsch MJ. Epidermal growth factor receptor expression is associated with rapid tumour cell proliferation in renal cell carcinoma. *Human Pathol* 1997; 28: 1255-9
- 32 Moch H, Sauter G, Gasser TC *et al*. EGF-r gene copy number changes in renal cell carcinoma detected by FISH. *J Pathol* 1998; 184: 424-9
- 33 Baselga J. Clinical trials of Herceptin (trastuzumab). *Eur J Can* 2001; 37: S18-24
- 34 Bartlett JMS, Hulme MJ, Miller WR. Analysis of Camp RI alpha mRNA expression in breast cancer. Evaluation of quantitative PCR for routine use. *Br J Cancer* 1996; 73: 1538-44
- 35 Yao M, Shuin T, Misaki H, Kubota Y. Enhanced expression of c-myc and epidermal growth factor receptor genes in primary human renal cell cancer. *Cancer Res* 1988; 48: 6753-7
- 36 Piccart M. Closing remarks and treatment guidelines. *Eur J Can* 2001; 37: 30-3

Authors

Z. Latif, ARCS(Ed), Research Fellow.
 A.D. Watters, MSc, Research Assistant.
 J.M.S. Bartlett, PhD, Senior Lecturer in Surgery.
 M.A. Underwood, MD, FRCS(Urol), Consultant Urologist.
 M. Aitchison, MD, FRCS(Urol), Consultant Urologist.
 Correspondence: Z. Latif, Department of Surgery, Queen Elizabeth Building, Glasgow Royal Infirmary, Glasgow, G31 2ER, UK.
 e-mail: zaklatif@hotmail.com

Abbreviations: FISH, fluorescent *in-situ* hybridization;
 IHC, immunohistochemistry.

Why the Epidermal Growth Factor Receptor? The Rationale for Cancer Therapy

JOSÉ BASELGA

Medical Oncology Service, Hospital Universitari Vall d'Hebron, Barcelona, Spain

Key Words: EGFR · Monoclonal antibodies · EGFR-TKIs · ZD1839 (Iressa®)

LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Explain the molecular biology of epidermal growth factor receptor (EGFR) function in malignant cells.
2. Recognize the relationships between and functions of the erbB family of related cell membrane receptors.
3. Describe the current status of clinical strategies to inhibit EGFR function in malignant cells.



Access and take the CME test online and receive one hour of AMA/PRA category 1 credit at CME.TheOncologist.com

ABSTRACT

There is a need for new, selective anticancer agents that differentiate between malignant and nonmalignant cells. The benefits of such agents would include a higher therapeutic index and lower toxicity than conventional therapies. Although expressed in nonmalignant cells, the epidermal growth factor receptor (EGFR) is highly expressed in a variety of tumors, and its expression correlates with poor response to treatment, disease progression, and poor survival. Evidence for a role for the EGFR in the inhibition and pathogenesis of various cancers has led to the rational design and development of agents that selectively target this receptor. Activation of the EGFR signaling pathway in cancer cells has been linked with increased cell proliferation, angiogenesis, and metastasis,

and decreased apoptosis. Preclinical data show that anti-EGFR therapies can inhibit these effects in vitro and in vivo. In addition, preclinical data confirm that many such agents have the potential to increase the effectiveness of current cytotoxic agents. Following accelerated drug development programs, phase III trials are now under way for a number of EGFR-targeted therapies, including the monoclonal antibody IMC-C225 and the EGFR-tyrosine kinase inhibitors ZD1839 (Iressa™) and OSI-774. Thus, the rationale for EGFR-targeted approaches to cancer treatment is apparent and now well established, and there is increasing evidence that they may represent a significant contribution to cancer therapy. *The Oncologist* 2002;7(suppl 4):2-8

INTRODUCTION

Over the past few decades, there has been considerable interest in developing new agents to improve the outcome for patients with solid tumors. However, traditional cytotoxic therapies are nonspecific and do not discriminate

between tumor and host cells [1]. Further, as they are generally effective against rapidly dividing neoplasms [2], their efficacy against solid tumors is limited. Even where cytotoxic agents are effective, tumor resistance may develop [3]. The lack of specificity and limited efficacy of traditional

Correspondence: José Baselga, M.D., Medical Oncology Service, Hospital General Universitari Vall d'Hebron, Paseo Vall d'Hebron 119-129, 08035 Barcelona, Spain. Telephone: 34-93-274-6077; Fax: 34-93-274-6059; e-mail: baselga@hg.vhebron.es Received June 28, 2002; accepted for publication July 25, 2002. ©AlphaMed Press 1083-7159/2002/\$5.00/0

The Oncologist 2002;7(suppl 4):2-8 www.TheOncologist.com

cytotoxic agents has led to the rational design and development of targeted therapies that aim to differentiate between malignant and nonmalignant cells, thereby producing a higher therapeutic index and less toxicity than conventional therapies [1]. In order to develop such agents, it is necessary to identify the aberrant biochemical and molecular pathways that distinguish malignant cells from nonmalignant cells [2]. As with nonmalignant cells, tumor growth and progression depend largely on the activity of cell membrane receptors that control the intracellular signal transduction pathways regulating cell proliferation and apoptosis, angiogenesis, adhesion, and motility [2].

One such cell membrane receptor is the epidermal growth factor receptor (EGFR), which has been shown to play an important role in the growth and survival of many solid tumors. Pathways involved in EGFR signal transduction have been proposed as possible anticancer targets, and agents to specifically target the EGFR have been developed [4-6].

EGFR AND SIGNALING PATHWAYS

The EGFR belongs to the erbB family of four closely related cell membrane receptors: EGFR (HER1 or erbB1), erbB2 (HER2), erbB3 (HER3), and erbB4 (HER4). These receptors are transmembrane glycoproteins that consist of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity for signal transduction (Fig. 1). Activation of the

EGFR occurs when a ligand, such as epidermal growth factor (EGF), transforming growth factor- α (TGF- α), or amphiregulin, binds to its extracellular domain. This causes the receptor to dimerize with either another EGFR monomer or with another member of the erbB family [7]. Following receptor dimerization, activation of the intrinsic protein tyrosine kinase activity and tyrosine autophosphorylation occur. These events lead to the recruitment and phosphorylation of several intracellular substrates, leading to mitogenic signaling and other cellular activities [8, 9]. Receptors that lack kinase function, because of mutations at the ATP binding site, do not display a full range of biochemical responses following ligand binding [10]; this demonstrates that receptor tyrosine kinase activity is required in cellular signaling. A major signaling route of the erbB family appears to be the *ras-raf*-mitogen-activated protein kinase pathway [8]. Another important pathway in erbB receptor signaling is the one constituted by phosphatidylinositol 3-kinase and the downstream protein kinase Akt [11, 12]. After its activation, Akt transduces signals that regulate multiple biological processes including apoptosis, gene expression, and cellular proliferation [13]. Akt is likely to send survival (antiapoptotic) signals by phosphorylating multiple targets, including the Bcl-2 family member BAD (a proapoptotic factor) [14] and the cell-death pathway enzyme caspase-9 [15]. Akt also plays a prominent role in regulation of cell cycle progression [13]. Thus, EGFR signaling can lead to a variety of downstream reactions, which are subject to complex regulatory mechanisms [16].

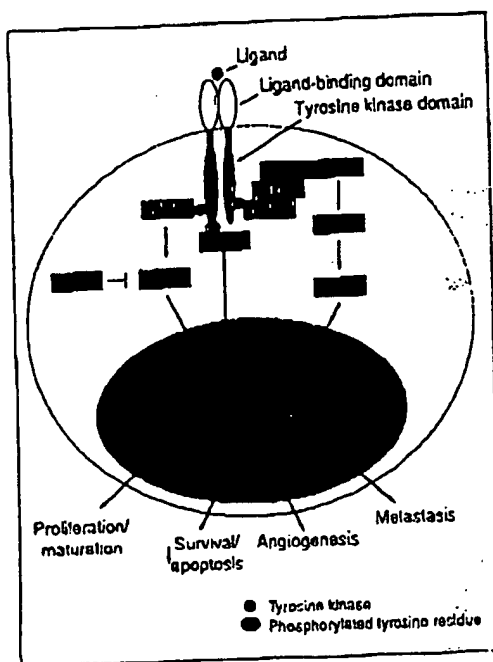


Figure 1. EGFR signal transduction. Adapted from [4] by permission from Signal 2000;1:12-21. ©2000 Adis International Ltd.

EGFR AND CANCER

EGFR signaling impacts on many aspects of tumor biology. Activation of the EGFR has been shown to enhance processes responsible for tumor growth and progression, including the promotion of proliferation, angiogenesis, and invasion/metastasis, and inhibition of apoptosis (Fig. 1) [4, 17, 18]. The expression of EGFR in tumors has been correlated with disease progression, poor survival, poor response to therapy [19], and the development of resistance to cytotoxic agents [20, 21]. High levels of EGFR have been observed in a variety of tumors, including prostate, breast, gastric, colorectal, and ovarian [4, 17, 22]. However, mechanisms other than EGFR expression affect EGFR signaling (reviewed by Arteaga pp. 31-37 [23]). For example, mutations in the EGFR are observed in some tumors; the most common mutant is EGFRvIII, which lacks an external ligand-binding domain and has a constitutively activated, but attenuated, tyrosine kinase [17]. EGFRvIII is commonly overexpressed as a result of gene amplification and has been identified in brain, lung, breast, prostate, and stomach cancers [6] but has not yet been found in nonmalignant cells.

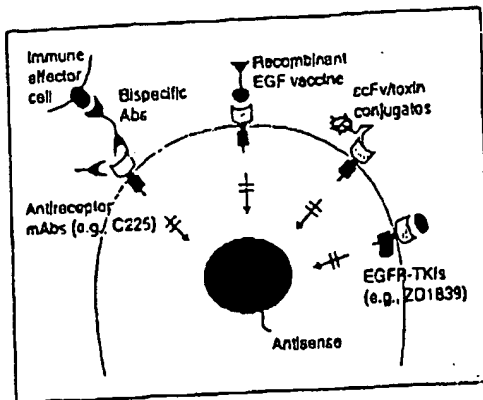


Figure 2. Strategies for EGF signaling inhibition. Adapted from [6] by permission from *Drugs* 2000, 60(suppl. 1):15-23. ©2000 Adis International Ltd. Abbreviations: EGF = epidermal growth factor; EGFR-TKI = epidermal growth factor receptor tyrosine kinase inhibitor; scFv = single-chain fragment variable.

CURRENT EGFR-TARGETED STRATEGIES

The clear potential for EGFR-targeted therapies in the treatment of cancer has prompted the development of a variety of agents targeted to the extracellular ligand-binding domain, the intracellular tyrosine kinase domain, the ligand, or to synthesis of the EGFR (Fig. 2). These agents are being investigated as monotherapy as well as in combination with conventional therapies.

A number of monoclonal antibodies (mAbs) directed against the extracellular ligand-binding domain, which prevent ligand binding (e.g., IMC-C225 and ABX-EGF), have been developed. Another approach is provided by bispecific antibodies (e.g., MDX-447) that target the extracellular ligand-binding domain of the EGFR as well as epitopes on the surface of immune effector cells, such as macrophage-activated killer cells. The aim is to encourage immune effector cell recruitment at the site of tumors, and hence, initiate destruction of the tumor cells and then stimulation of additional immune responses. Single-chain fragment variable (scFv) antibodies against the EGFR conjugated to toxins, such as pseudomonas endotoxin A (ETA), as well as to fungal and plant-derived toxins, have also been investigated [24]. One of the most potent conjugates is the scFv-14e1-ETA-fusion toxin, which binds to EGFR and EGFRvIII with equal affinity but has 100-fold enhanced cytotoxicity against tumors expressing EGFRvIII compared with those expressing EGFR [25, 26].

Another approach has been to target the intracellular tyrosine kinase domain of the EGFR using small-molecule EGFR tyrosine kinase inhibitors (EGFR-TKIs), such as ZD1839 (Iressa™) and OSI-774. These inhibit ATP binding to the tyrosine kinase domain of the receptor, thereby inhibiting tyrosine

kinase activity and autophosphorylation, and subsequently, blocking signal transduction from the EGFR.

Additional tactics used to target EGFR signaling have been directed against its ligands, such as the recombinant EGF vaccine, EGF-P64k, which consists of recombinant human EGF conjugated to a highly immunogenic recombinant bacterial protein P64k [27]. Therapies that target both ligand and EGFR production are also being investigated using antisense oligonucleotides to block the translation of the TGF- α and EGFR genes into their respective proteins.

Of these EGFR-targeted agents, the mAb IMC-C225 and the EGFR-TKIs ZD1839 and OSI-774 are the furthest developed (Table 1). Preclinical data from these agents support the rationale for the use of EGFR-targeted agents in cancer therapy, and their potential is being evaluated in clinical trials.

EGFR-TARGETED AGENTS IN CANCER: PRECLINICAL VALIDATION

Both in vitro and in vivo studies have demonstrated that EGFR-targeted agents inhibit the processes involved in tumor growth and progression, including proliferation, apoptosis, metastasis, and angiogenesis. To illustrate the rationale for targeting the EGFR, two agents that operate using different mechanisms are described below: the mAb IMC-C225 and the EGFR-TKI ZD1839.

Table 1. EGFR-targeted strategies and their development stages

Class of compound	Name	Development stage
mAbs	IMC-C225	phase III
	ABX-EGF	phase II
	EMD-72060	phase II
	TheraCIM-h-R3	phase II
	mAb-806	preclinical
Bispecific antibodies	MDX-447	phase II
EGFR-TKIs		
	Quinoxalines	
	ZD1839	phase III
	OSI-774	phase III
	CI-1033	phase II
Pyridopyrimidines	EKB-569	phase I
	PD-0183805	phase I
	PD-158780 series	preclinical
	PD-180970	preclinical
	Pyrolopyrimidines	phase I
Other compounds	PKI-166	phase I
	GW-572016/GW-2016	phase I
	LFM-A12	preclinical
Recombinant vaccine	EGF-P64k	phase II
Antisense oligonucleotides	AS-21	preclinical

The anti-EGFR mAb IMC-C225 (cetuximab) has been shown to inhibit cell growth and survival in vitro and in vivo [28]. It causes an increase in the expression of the cell cycle inhibitor p27^{Kip1}, resulting in the formation of inhibitory p27^{Kip1}-cyclin-dependent kinase-2 complexes that prevent cells from exiting the G₁ phase of the cell cycle [29]. IMC-C225 has also been shown to induce apoptosis in some cell lines [30] and to inhibit the production of angiogenic factors, in vitro and in vivo [31], as well as metastasis [32].

Data from in vitro studies have revealed that, in addition to reducing cell proliferation, the EGFR-TKI ZD1839 induces cell cycle arrest, increases apoptosis, and has anti-angiogenic activity [3, 33]. In addition, ZD1839 has been shown to have antimetastatic properties in human head and neck and breast cancer cells [34]. ZD1839 inhibited cancer cell migration and invasiveness by blocking p21-activated kinase 1, which is vital for directional motility and cell survival. In vivo studies have confirmed its ability to inhibit tumor growth in a variety of tumor types including prostate, breast, ovarian, colon, small-cell lung, and non-small cell lung cancer (NSCLC) [33, 35, 36]. However, the level of expression of the EGFR in xenografts does not seem to influence the effect of ZD1839, indicating that the level of expression of the EGFR is not the only factor to influence EGFR signaling [37].

Preclinical studies have also demonstrated that EGFR-targeted agents have potential for use in combination with cytotoxic chemotherapy and with radiotherapy. IMC-C225 has been shown to enhance the effects of cytotoxic agents [38-40] and radiotherapy [41, 42]; for example, IMC-C225 in combination with topotecan increased survival of nude mice bearing human colon cancer xenografts [39]. ZD1839 also potentiated the growth-inhibitory effects of cytotoxic agents [35, 36, 43, 44], and preliminary data indicate additive or synergistic effects in combination with ionizing radiation [43].

EGFR-TARGETED AGENTS IN CANCER: CLINICAL VALIDATION

The use of mAbs against the erbB family of receptors has been validated with trastuzumab, a humanized mAb raised against the extracellular domain of erbB2, which gained U.S. Food and Drug Administration (FDA) approval in September 1998 for the treatment of metastatic breast cancer. Trastuzumab is generally well tolerated, although serious cardiac side effects may occur in some patients [45], especially those aged over 60 years or those receiving concomitant doxorubicin/ cyclophosphamide. The cardiotoxicity of trastuzumab is currently not well understood and is under intense scrutiny; it may be related to cardiac expression of erbB2 [46].

The most advanced anti-EGFR mAb in clinical development is IMC-C225. The recently reported preliminary results of the following trials have been promising: a phase III trial of IMC-C225 in combination with cisplatin in patients with metastatic or recurrent head and neck cancer [47]; a phase II trial of IMC-C225 monotherapy in colorectal cancer [48]; and phase I/II trials of combination therapy with cisplatin [49] or cisplatin/carboplatin [50] in squamous-cell carcinoma of the head and neck and with irinotecan/5-fluorouracil/leucovorin in colorectal cancer [51]. The most common adverse event related to IMC-C225 was acneiform rash.

Although the chimeric antibody IMC-C225, formed by replacing the constant region of the original mouse mAb with the constant region of a human immunoglobulin, greatly reduces immunogenicity compared with the original mouse mAb, anaphylactic reactions and loss of efficacy have been seen after repeated exposure, due to the formation of human-antimouse antibodies [46]. A humanized version of IMC-C225, EMD-72000, has, therefore, been developed and, following promising efficacy as a single agent in phase I trials, is under evaluation in phase II trials in patients with ovarian and head and neck cancers [52].

The potential of therapies targeting tyrosine kinases has been demonstrated with imatinib, an inhibitor of tyrosine kinases associated with Bcr-Abl and c-kit and the platelet-derived growth factor receptor tyrosine kinase. Imatinib was launched in the U.S. in May 2001 for the treatment of patients with chronic myeloid leukemia. In February 2002, imatinib gained FDA approval for use in patients with inoperable and/or metastatic malignant gastrointestinal stromal tumors.

Clinical trials have shown that ZD1839 is active in solid tumors, with the most common side effects being mild, reversible rash and diarrhea (reviewed by *Herbst* [53], *Natale* [54], and *Ranson* [55] in this issue). ZD1839 is currently undergoing phase III evaluation in combination with other cytotoxic agents in NSCLC, having demonstrated clinically meaningful activity in phase II trials in patients with head and neck cancer [56] and NSCLC [57, 58], reviewed by *Herbst* in this issue [53]. In addition to the expected antiproliferative effect of EGFR-TKIs, resulting in disease stabilization, partial responses were observed in some patients. Phase I trials have also shown that the combination of ZD1839 with other cytotoxic agents is feasible [59, 60], reviewed by *Ranson* in this issue [55].

Early trials suggest that OSI-774 monotherapy has some activity in NSCLC, head and neck cancer, and ovarian carcinoma [61-63]; combination studies are also under way [64, 65]. Preliminary phase I studies of OSI-774 in combination with standard chemotherapeutic agents, such

as docetaxel, gemcitabine plus cisplatin, and carboplatin plus paclitaxel, have shown no major interactions among OSI-774 and these drugs [64, 66]. A phase III study of OSI-774 with gemcitabine plus cisplatin, using the drug regimen determined in phase I trials, is currently ongoing in Europe in patients with NSCLC [66], and a similar study with carboplatin and taxel is under way in the U.S.

CONCLUSION

As the EGFR is highly expressed in a variety of solid tumors and is associated with poor response to treatment, disease progression, and poor survival, EGFR inhibition is a logical anticancer strategy. Many potential points of intervention on the receptor have been identified, and mechanisms include inhibition of ligand binding and intracellular signaling. Preclinical results from a multitude of novel anti-EGFR agents have shown that many of the approaches to inhibit EGFR signaling are feasible and that

inhibition of the EGFR causes cancer cell proliferation, angiogenesis, and metastasis to decrease, and apoptosis to increase. Many of the intracellular pathways involved with these anticancer effects are being probed; this understanding has led to the development of many agents, potentially benefiting patients with a variety of tumors. The clinically furthest developed: IMC-C225, ZD1839, and OSI-774 are currently undergoing phase III evaluation. In addition to efficacy as monotherapy, these agents successfully enhance the activity of conventional cytotoxic agents and may provide alternative treatment regimens to patients with solid tumors.

ACKNOWLEDGMENT

Dr. Jose Baselga is a consultant for AstraZeneca and Roche, and receives grant and research support from Bristol-Myers Squibb. At the time of publication, this paper discusses the unlabeled usage of ZD1839 and IMC-225.

REFERENCES

- 1 Rowinsky EK. The pursuit of optimal outcomes in cancer therapy in a new age of rationally designed target-based anticancer agents. *Drugs* 2000;60(suppl 1):1-14.
- 2 Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.
- 3 Ciardiello F, Tortora G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. *Clin Cancer Res* 2001;7:2958-2970.
- 4 Baselga J. New technologies in epidermal growth factor receptor-targeted cancer therapy. *Signal* 2000;1:12-21.
- 5 Goel S, Mani S, Perez-Soler R. Tyrosine kinase inhibitors: a clinical perspective. *Curr Oncol Rep* 2002;4:9-19.
- 6 Raymond E, Faivre S, Armand JP. Epidermal growth factor receptor tyrosine kinase as a target for anticancer therapy. *Drugs* 2000;60(suppl 1):15-23.
- 7 Sako Y, Minoguchi S, Yanagida T. Single-molecule imaging of EGFR signalling on the surface of living cells. *Nat Cell Biol* 2000;2:168-172.
- 8 Alroy I, Yarden Y. The ErbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand-receptor interactions. *FEBS Lett* 1997;410:83-86.
- 9 Riese 2nd DJ, Stern DF. Specificity within the EGF family/ErbB receptor family signaling network. *Bioessays* 1998;20:41-48.
- 10 Chen WS, Lazar CS, Poenie M et al. Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature* 1987;328:820-823.
- 11 Burgering BM, Coffey PJ. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 1995;376:599-602.
- 12 Muthuswamy SK, Gilman M, Brugge JS. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. *Mol Cell Biol* 1999;19:6845-6857.
- 13 Chan TO, Rittenhouse SE, Tsichlis PN. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* 1999;68:965-1014.
- 14 Datta SR, Dudek H, Tao X et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* 1997;91:231-241.
- 15 Cardone MH, Roy N, Stennicke HR et al. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 1998;282:1318-1321.
- 16 Moghal N, Stenberg PW. Multiple positive and negative regulators of signaling by the EGF-receptor. *Curr Opin Cell Biol* 1999;11:190-196.
- 17 Wells A. The epidermal growth factor receptor (EGFR)—a new target in cancer therapy. *Signal* 2000;1:4-11.
- 18 Woodburn JR. The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther* 1999;82:241-250.
- 19 Brabender J, Danenberg KD, Metzger R et al. Epidermal growth factor receptor and HER2-neu mRNA expression in non-small cell lung cancer is correlated with survival. *Clin Cancer Res* 2001;7:1850-1855.
- 20 Meyers MB, Shen WP, Spengler BA et al. Increased epidermal growth factor receptor in multidrug-resistant human neuroblastoma cells. *J Cell Biochem* 1988;38:87-97.
- 21 Wosikowski K, Schuurhuis D, Kops GJ et al. Altered gene expression in drug-resistant human breast cancer cells. *Clin Cancer Res* 1997;3:2405-2414.
- 22 Salomon DS, Brandt R, Ciardiello F et al. Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit Rev Oncol Hematol* 1995;19:183-232.

- 23 Arteaga C. Epidermal growth factor receptor dependence in human tumors: more than just expression? *The Oncologist* 2002;7(suppl 4):31-39.
- 24 Noonberg SB, Benz CC. Tyrosine kinase inhibitors targeted to the epidermal growth factor receptor subfamily: role as anticancer agents. *Drugs* 2000;59:753-767.
- 25 Schmidt M, Reiser P, Hills D et al. Expression of an oncogenic mutant EGF receptor markedly increases the sensitivity of cells to an EGF-receptor-specific antibody-toxin. *Int J Cancer* 1998;75:878-884.
- 26 Schmidt M, Maurer-Gebhard M, Groner B et al. Suppression of metastasis formation by a recombinant single chain antibody-toxin targeted to full-length and oncogenic variant EGF receptors. *Oncogene* 1999;18:1711-1721.
- 27 Gonzalez G, Crombet T, Catala M et al. A novel cancer vaccine composed of human-recombinant epidermal growth factor linked to a carrier protein: report of a pilot clinical trial. *Ann Oncol* 1998;9:431-435.
- 28 Mendelsohn J, Baselga J. The EGF receptor family as targets for cancer therapy. *Oncogene* 2000;19:6550-6565.
- 29 Peng D, Fan Z, Lu Y et al. Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates p27^{kat5} and induces G₁ arrest in prostatic cancer cell line DU145. *Cancer Res* 1996;56:3666-3669.
- 30 Wu X, Fan Z, Masui H et al. Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest* 1995;95:1897-1905.
- 31 Petit AM, Rak J, Hung MC et al. Neutralizing antibodies against epidermal growth factor and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells: in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am J Pathol* 1997;151:1523-1530.
- 32 Perrotte P, Matsumoto T, Inoue K et al. Anti-epidermal growth factor receptor antibody C225 inhibits angiogenesis in human transitional cell carcinoma growing orthotopically in nude mice. *Clin Cancer Res* 1999;5:257-265.
- 33 Ciardiello F, Caputo R, Bianco R et al. Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 2001;7:1459-1465.
- 34 Mandal M, Adam L, Wang R-A et al. Inhibition of p21-activated kinase 1, directional cell motility and invasion of growth-factor-activated human cancer cells by the selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) ZD1839 ('Iressa'). *Proc Am Assoc Cancer Res* 2002;43:A786.
- 35 Ciardiello F, Caputo R, Bianco R et al. Antitumor effect and potentiation of cytotoxic drugs activity in human cancer cells by ZD-1839 (Iressa), an epidermal growth factor receptor-selective tyrosine kinase inhibitor. *Clin Cancer Res* 2000;6:2053-2063.
- 36 Sirounak F, Zakowski MF, Miller VA et al. Potentiation of cytotoxic agents against human tumors in mice by ZD1839 ('Iressa'), an inhibitor of EGFR tyrosine kinase, does not require high levels of expression of EGFR. *Proc Am Assoc Cancer Res* 2000;41:482a.
- 37 Woodburn J, Kendrew J, Fennell M et al. ZD1839 ('Iressa') a selective epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI): inhibition of c-fos mRNA an intermediate marker of EGFR activation, correlates with tumor growth inhibition. *Proc Am Assoc Cancer Res* 2000;41:402a.
- 38 Baselga J, Norton L, Masui H et al. Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. *J Natl Cancer Inst* 1993;85:1327-1333.
- 39 Ciardiello F, Bianco R, Damiano V et al. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. *Clin Cancer Res* 1999;5:909-916.
- 40 Kim ES, Khuri FR, Herbst RS. Epidermal growth factor receptor biology (IMC-C225). *Curr Opin Oncol* 2001;13:506-513.
- 41 Huang SM, Harari PM. Modulation of radiation response after epidermal growth factor receptor blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. *Clin Cancer Res* 2000;6:2166-2174.
- 42 Carpenter M, Buchsbaum DJ. Statistical growth curve modeling of tumors treated with ERBITUX™ (IMC-C225) anti-EGFR antibody, gemcitabine, and radiation. *Proc Am Assoc Cancer Res* 2002;43:A2386.
- 43 Raben D, Hellrich BA, Chan D et al. ZD1839, a selective epidermal growth factor receptor tyrosine kinase inhibitor, alone and in combination with radiation and chemotherapy as a new therapeutic strategy in non-small cell lung cancer. *Semin Oncol* 2002;29(suppl 4):37-46.
- 44 Ciardiello F, Caputo R, Damiano V et al. Potentiation of cytotoxic drug activity in human cancer cells by ZD1839 ('Iressa') an EGFR-selective tyrosine kinase inhibitor. *Proc Am Assoc Cancer Res* 2000;41:11a.
- 45 Seidman A, Hudis C, Pierri MK et al. Cardiac dysfunction in the trastuzumab clinical trials experience. *J Clin Oncol* 2002;20:1215-1221.
- 46 Slichenmyer WJ, Fry DW. Anticancer therapy targeting the erbB family of receptor tyrosine kinases. *Semin Oncol* 2001;28(suppl 16):67-79.
- 47 Burness BA, Li Y, Flood W et al. Phase III trial comparing cisplatin (C) + placebo (P) to C + anti-epidermal growth factor antibody (EGF-R) C225 in patients (pts) with metastatic/recurrent head & neck cancer (HNC). *Proc Am Soc Clin Oncol* 2002;21:226a.
- 48 Saltz L, Meropol NJ, Loehrer PJ et al. Single agent IMC-225 (Erbix™) has activity in CPT-11-refractory colorectal cancer (CRC) that expresses the epidermal growth factor receptor (EGFR). *Proc Am Soc Clin Oncol* 2002;21:127a.
- 49 Kies MS, Arquette MA, Nabell L et al. Final report of the efficacy and safety of the anti-epidermal growth factor antibody Erbitux (IMC-C225), in combination with cisplatin in patients with recurrent squamous cell carcinoma of the head and neck (SCCHN) refractory to cisplatin containing chemotherapy. *Proc Am Soc Clin Oncol* 2002;21:232a.

- 50 Baselga J, Trigo JM, Bourhis J et al. Cetuximab (C225) plus cisplatin/carboplatin is active in patients (pts) with recurrent/metastatic squamous cell carcinoma of the head and neck (SCCHN) progressing on a same dose and schedule platinum-based regimen. *Proc Am Soc Clin Oncol* 2002;21:226a.
- 51 Rosenberg AH, Lochrer PJ, Needle MN et al. Erbitux (IMC-C225) plus weekly irinotecan (CPT-11), fluorouracil (5FU), and leucovorin (LV) in colorectal cancer (CRC) that expresses the epidermal growth factor receptor (EGFR). *Proc Am Soc Clin Oncol* 2002;21:135a.
- 52 Tewes M, Schleucher N, Dirsch O et al. Results of a phase I trial of the humanized anti-epidermal growth factor receptor (EGFR) monoclonal antibody BMD 72000 in patients with EGFR-expressing solid tumors. *Proc Am Soc Clin Oncol* 2002;21:95a.
- 53 Herbst RS. ZD1839 (Iressa) in non-small-cell lung cancer. *The Oncologist* 2002;7(suppl 4):9-15.
- 54 Natale RB. ZD1839 (Iressa): what's in it for the patient? *The Oncologist* 2002;7(suppl 4):25-30.
- 55 Ranson M. ZD1839 (Iressa): for more than just non-small-cell lung cancer. *The Oncologist* 2002;7(suppl 4):16-24.
- 56 Cohen EEW, Rosen F, Dekker A et al. Phase II study of ZD1839 ('Iressa') in recurrent or metastatic squamous cell carcinoma of the head and neck (SCCHN). *Proc Am Soc Clin Oncol* 2002;21:225a.
- 57 Kris MG, Natale RB, Herbst RS et al. A phase II trial of ZD1839 ('Iressa') in advanced non-small cell lung cancer (NSCLC) patients who had failed platinum- and docetaxel-based regimens (IDEAL 2). *Proc Am Soc Clin Oncol* 2002;21:292a.
- 58 Fukuoka M, Yano S, Giaccone G et al. Final results from a phase II trial of ZD1839 ('Iressa') for patients with advanced non-small-cell lung cancer (IDEAL 1). *Proc Am Soc Clin Oncol* 2002;21:298a.
- 59 Braun AH, Dirsch O, Hilger R-A et al. Preclinical evaluation of the combination of epidermal growth factor inhibitor ZD1839 (Iressa) and irinotecan (SN-38) in human colon cancer cells. *Proc Am Soc Clin Oncol* 2002;21:83a.
- 60 Cho CD, Fisher GA, Halsety JZ et al. A phase I study of ZD1839 (Iressa) in combination with oxaliplatin, 5-fluorouracil (5-FU) and leucovorin (LV) in advanced solid malignancies. *Proc Am Soc Clin Oncol* 2002;21:10a.
- 61 Perez-Soler R, Chachoua A, Huberman M et al. A phase II trial of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor OSI-774 following platinum-based chemotherapy, in patients (pts) with advanced, EGFR-expressing, non-small cell lung cancer (NSCLC). *Proc Am Soc Clin Oncol* 2001;20:310a.
- 62 Senzer NN, Soulières D, Siu L et al. Phase 2 evaluation of OSI-774, a potent oral antagonist of the EGFR-TK in patients with advanced squamous cell carcinoma of the head and neck. *Proc Am Soc Clin Oncol* 2001;20:2a.
- 63 Finkler N, Gordon A, Crozier M et al. Phase 2 evaluation of OSI-774, a potent oral antagonist of the EGFR-TK in patients with advanced ovarian carcinoma. *Proc Am Soc Clin Oncol* 2001;20:208a.
- 64 Forouzesht B, Hidalgo M, Takimoto C et al. Phase I, pharmacokinetic (PK), and biological studies of the epidermal growth factor-tyrosine kinase (EGFR-TK) inhibitor OSI-774 in combination with docetaxel. *Proc Am Soc Clin Oncol* 2002;21:21a.
- 65 Forero L, Patnaik A, Hammond LA et al. Phase I, pharmacokinetic (PK) and biologic study of OSI-774, a selective epidermal growth factor receptor (EGFR) tyrosine kinase (TK) inhibitor in combination with paclitaxel and carboplatin. *Proc Am Soc Clin Oncol* 2002;21:25b.
- 66 Ratain MJ, George CM, Janisch L et al. Phase I trial of erlotinib (OSI-774) in combination with gemcitabine (G) and cisplatin (P) in patients with advanced solid tumors. *Proc Am Soc Clin Oncol* 2002;21:76b.

Cutaneous side-effects in cancer patients treated with the antiepidermal growth factor receptor antibody C225

K.J.BUSAM, P.CAPODIECI, R.MOTZER,* T.KIEHN,† D.PHELAN* AND A.C.HALPERN*

Departments of Pathology, *Medicine and †Laboratory Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, U.S.A.

Accepted for publication 25 January 2001

Summary

Background C225 is an antibody to the epidermal growth factor receptor (EGFR), and inhibits growth of various tumour cells. The antibody is currently being used as a therapeutic agent in several clinical trials of patients with carcinomas.

Objectives To determine and investigate the cutaneous side-effects in cancer patients treated with C225.

Methods We clinically examined 10 patients treated with C225, and performed immunohistochemical and *in situ* hybridization studies on skin biopsies.

Results The most common cutaneous reaction to C225 therapy was the development of an acneiform follicular eruption, which was most pronounced on the face, chest and upper back and typically manifested a week after the onset of treatment. The consistency of the morphology and timing of the clinical findings in 10 different patients following monotherapy with C225 strongly suggested a direct biological effect of the antibody. Additional dermatological side-effects included focal areas of tender paronychia inflammation of toes and fingers and small aphthous ulcers of the oral mucosa. Serial punch biopsies of chest skin before and after treatment (at 8 days) revealed two main reaction patterns: a superficial dermal inflammatory cell infiltrate surrounding hyperkeratotic and ectatic follicular infundibula, and a suppurative superficial folliculitis. In two biopsies focal intraepidermal acantholysis was found. Microbiological cultures failed to reveal an infectious aetiology. Immunohistochemical and *in situ* hybridization studies on a subset of the biopsies showed an increase in the expression of p27^{Kip1} in epidermal keratinocytes after treatment with C225.

Conclusions Our findings support the concept that p27^{Kip1} plays a part in the *in vivo* regulation of follicular and epidermal homeostasis by EGFR.

Key words: C225, epidermal growth factor receptor, folliculitis

C225 is an antibody to the epidermal growth factor (EGF) receptor (EGFR), and has previously been shown to block the proliferation of various cancer cells.^{1–4} It is currently being investigated in several clinical trials of patients with cancer of the head and neck, breast, lung, prostate and kidney as a single therapeutic agent or in combination with other drugs.^{5–7} Cutaneous side-effects are common in these patients, but have not been well characterized and the underlying mechanisms have not been investigated.

We studied the cutaneous findings of 10 patients

with renal cell carcinoma who participated in a treatment protocol with C225 as a single agent. The patients were followed clinically and punch biopsies were taken at regular intervals from chest skin. We present our findings of the clinical appearance of the lesions and show the pathological findings of the biopsies.

Materials and methods

Following written informed consent, a series of 10 patients (nine men and one woman; age range 47–76 years, mean 58.4) enrolled in a non-randomized phase

Correspondence: Klaus J. Busam.
E-mail: busamk@mskcc.org

II clinical trial of C225 antibody therapy for metastatic renal cell carcinoma at the Memorial Sloan-Kettering Cancer Center (New York) were studied with baseline and weekly dermatological evaluation and photography. Patients who had received prior chemotherapy or immunotherapy were excluded from the study. Study participants received C225 by intravenous infusion weekly at an initial loading dose of 500 mg m^{-2} followed by seven consecutive weekly doses at 250 mg m^{-2} . A baseline questionnaire documented Fitzpatrick skin type, past or recent history of acne, and all topical and systemic medications being used at time of study entry.

The severity of the cutaneous reactions was graded at weekly follow-up visits as: grade 1, asymptomatic macular or papular erythematous eruption in an acneiform distribution; grade 2, same as 1, but with symptoms such as pruritus; grade 3, extension of the eruption beyond the acneiform distribution of head, chest and back or the development of confluent lesions, painful lesions, or minor ulceration; grade 4, exfoliative or ulcerating dermatitis.

Surveillance bacterial cultures of forehead skin were performed at baseline and day 8. Following a gentle cleansing of the forehead, a sterile PVM/MA copolymer-impregnated strip (Chesebrough-Pond's, NJ, U.S.A.) was applied to the skin for 5 min and the stratum corneum and follicular contents stripped from the skin. The strip contents were suspended in an aliquot of bacterial medium and plated on bacterial and fungal media.

Serial 4-mm punch biopsies of chest skin were performed immediately prior to the initial infusion, 1 h following completion of the initial infusion, and on day 8 of therapy. In addition, several of the patients underwent a skin biopsy on day 3 or 4 of therapy. The biopsies were kept fresh in saline. Upon arrival in the pathology laboratory (Memorial Sloan-Kettering Cancer Center, New York), the biopsies were split in half. One half was frozen, the other routinely processed, i.e. fixed in 10% formalin and embedded in paraffin. Multiple haematoxylin and eosin-stained sections per biopsy were examined and Gram as well as periodic acid-Schiff-D stains were obtained to assess the presence or absence of bacteria and fungi. For cases that lacked a follicle or had only minimal inflammation on initial sectioning, the tissue block was extensively step-sectioned in search of a follicle and/or more prominent inflammation.

Immunohistochemistry was performed on biopsies from four patients whose tissue block contained sufficient material for such an analysis. The avidin-biotin method

was used with the monoclonal antibody against p27^{Kip1} (Ab-2; Oncogene Science, Inc., Boston, MA, U.S.A.; $0.1 \mu\text{g mL}^{-1}$ final concentration) as previously described,^{8,9} and UCHL1 (pan-T-cell marker; Dako; 1 : 200). Endogenous peroxidase was suppressed by a 20-min incubation with 1% H_2O_2 . Labelling of the secondary antibody was performed with an avidin-biotin complex using a biotinylated horse antimouse secondary (Vector, Burlingame, CA, U.S.A.; 1 : 200 dilution) and diaminobenzidine tetrahydrochloride (Biogenex, San Ramon, CA, U.S.A.) as a chromogen. The number of stained nuclei was counted per high-power microscope field (HPF; $\times 40$ objective). Three HPFs were examined per biopsy.

In situ hybridization studies for p27^{Kip1} mRNA expression were carried out as previously described.^{8,9} Digoxigenin-labelled probes were used and $1 \mu\text{g}$ of recombinant plasmid pCRTMII (Invitrogen Corp., San Diego, CA, U.S.A.), containing the full-length human p27KIP1 gene (a gift from Dr M. Pagano, New York University School of Medicine), was linearized by *Bam*HI and *Xba*I to generate anti-sense and sense transcripts. Tissue sections were rinsed in water and phosphate-buffered saline (PBS) for 10 min. The slides were digested with proteinase K ($50 \mu\text{g mL}^{-1}$) for 18 min at 37°C in PBS and postfixed at 4°C in a freshly prepared solution of 4% paraformaldehyde in PBS for 5 min. Prehybridization was for 30 min at 45°C in 50% formamide and $2 \times$ standard saline citrate (SSC). The hybridization buffer contained 50% (v/v) deionized formamide, 10% dextran sulphate (50% stock solution), $2 \times$ SSC ($20 \times$ stock solution), 1% sodium dodecyl sulphate (10% stock solution) and herring sperm DNA ($0.25 \mu\text{g mL}^{-1}$). Hybridization was performed overnight at 45°C by applying digoxigenin-labelled RNA probe (10 pmol L^{-1} in $50 \mu\text{L}$ of hybridization buffer per section) under a coverslip. The coverslips were removed, and the slides were washed in prewarmed $2 \times$ SSC for 20 min at 60°C twice, followed by washes in prewarmed $0.5 \times$ SSC and $0.021 \times$ SSC each at 60°C for 20 min. After these washes, the slides were incubated in normal sheep serum diluted in buffer at pH 7.5 and successively in the same buffer with antidigoxigenin antibody conjugated to alkaline phosphatase (Roche Diagnostics, IN, USA) at a dilution of 1 : 1500 for 1 h at room temperature. The visualization was accomplished by use of nitroblue tetrazolium 5-bromo-4-chloro-3-indolyl phosphate. The slides were counterstained with methyl green and mounted.

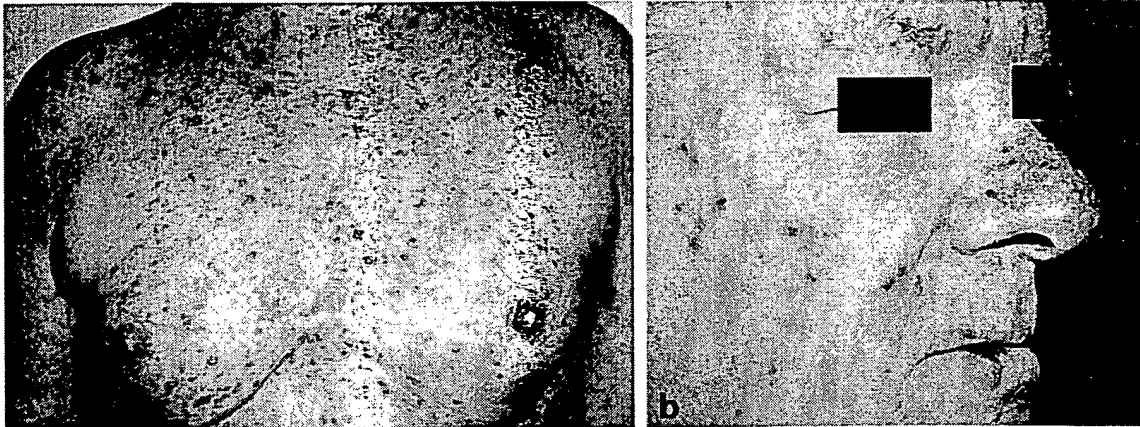


Figure 1. Clinical spectrum of the follicular rash. (a) Involvement of the skin of the chest (patient 3). (b) Involvement of the skin of the face (patient 2).

Results

Clinical findings

The most common side-effect of C225 therapy was a follicular rash, which commonly began in an acneiform distribution (face, scalp, chest and upper back) approximately 1 week into therapy (Fig. 1). It developed in all 10 patients. The time course of the clinical severity of the rash is depicted in Figure 2. There was no apparent association between severity of the rash and skin type or history of acne. Empirical use of topical and systemic antibiotics as well as topical steroids was not associated with consistent clinical improvement of the rash.

Another common dermatological manifestation in our patients was pain, tenderness and fissuring of the distal finger tufts that occurred in varying degrees in all of the patients. Five patients developed paronychia inflammation with associated swelling of the lateral nail folds of toes and fingers (Fig. 3). The digits most commonly affected were the great toes and thumbs. In one patient the middle finger was affected as well. In several instances the lateral nail fold swelling was associated with friable pyogenic granuloma-like

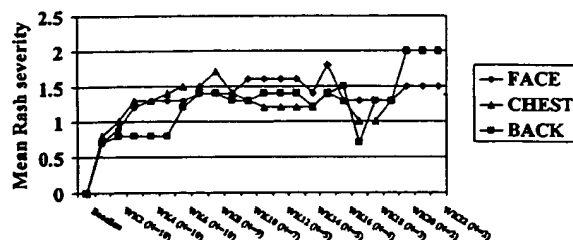


Figure 2. Severity of the rash over time by site.

changes that bled with minimal trauma. There was no preceding history of ingrown nails. Neither *Candida* nor bacteria were found on culture at the onset of these lesions. *Staphylococcus aureus* was cultured from some lesions, and persisted despite antibiotic therapy. Greatest symptomatic relief was achieved with soaks and cushioning of affected areas.

Two patients reported minimally symptomatic sores of the oral mucosa during therapy. They were found to have a few 2–3 mm intraoral aphthous ulcers. Herpesvirus direct immunofluorescence and culture were negative in these patients.

Histological findings

The histological findings in serial punch biopsies performed on chest skin from nine patients treated

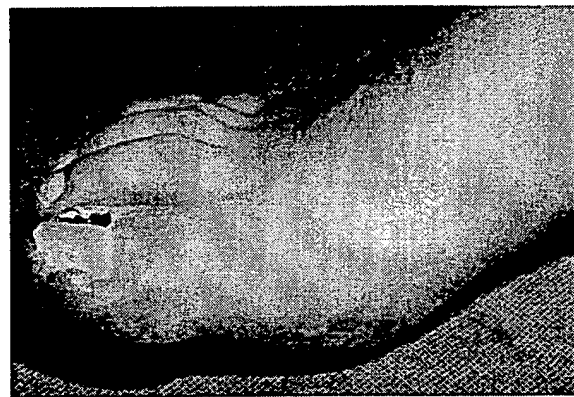


Figure 3. Paronychia swelling and friability of the lateral nail fold of the great toe (patient 2).

Patient	Day of treatment	Histological findings
1	1	Normal skin
	8	Suppurative folliculitis
2	1	Normal skin
	3	Superficial perifolliculitis
	8	Suppurative superficial folliculitis
3	1	Normal skin
	4	Suppurative folliculitis
	8	Suppurative folliculitis
4	1	Normal skin
	8	Perivascular dermatitis (no follicle present)
5	1	Normal skin
	4	Suppurative folliculitis; focal intraepidermal acantholysis
	8	Suppurative folliculitis
6	1	Normal skin
	8	Focal intraepidermal acantholysis and mild perifolliculitis
7	1	Normal skin
	8	Superficial perifolliculitis
8	1	Normal skin
	8	Superficial perifolliculitis
9	1	Normal skin
	8	Superficial perifolliculitis

Table 1. Summary of histological findings**Figure 4.** Superficial dermal infiltrate of lymphocytes surrounding a hyperkeratotic follicular infundibulum (haematoxylin and eosin; original magnification $\times 200$).

with C225 are summarized in Table 1. Before treatment with C225 was initiated, a control biopsy of normal skin was obtained, which was unremarkable in all cases, except for the presence of rare incidental *Pityrosporum* organisms in the stratum corneum of three patients. The subsequent biopsies showed variable inflammatory changes. The earliest findings included an infiltrate of T lymphocytes (immunoreactive for CD45RO; not shown) surrounding the follicular infundibulum (Fig. 4). After 1 week of treatment, four patients had a superficial perifolliculitis involving hyperkeratotic and ectatic follicular infundibula (Table 1), four had a florid suppurative folliculitis (Fig. 5), and two showed focal intraepidermal acantholysis in association with a sparse neutrophilic infiltrate involving the terminal portion of the sweat duct (Fig. 6). In one patient, the biopsy obtained at day 8 lacked a follicle upon serial sectioning. Thus, evaluation of follicular or perifollicular inflammation was not possible in this case.

Table 2. Immunoreactivity for p27^{Kip1}

Patient	No. of stained nuclei per high-power microscope field	
	Prior to treatment	Day 8 of treatment
1	6	20
2	8	25
3	7	32
5	12	35

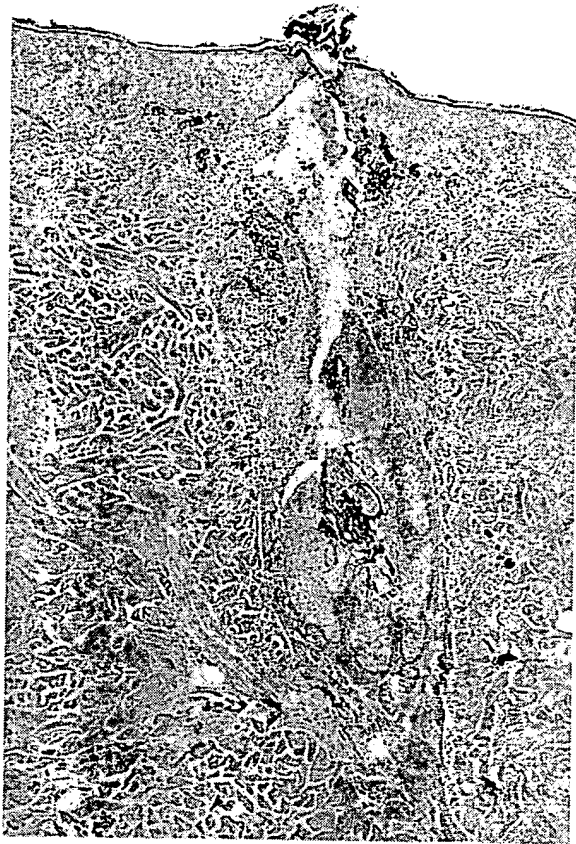


Figure 5. Suppurative superficial folliculitis. A hair follicle is involved by a florid mixed inflammatory cell infiltrate and rupture of the epithelial lining is present (haematoxylin and eosin; original magnification $\times 40$).

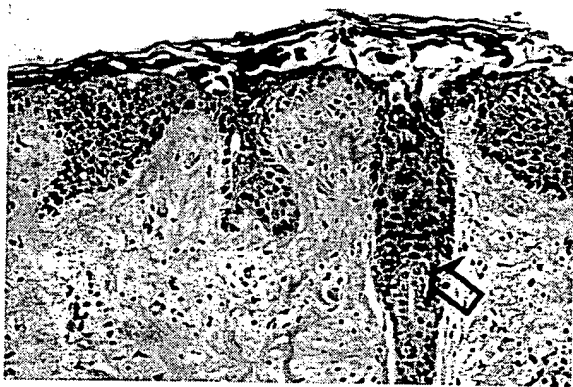


Figure 6. Intraepidermal acantholysis overlying the terminal portion of a sweat duct, which is focally involved by a neutrophilic infiltrate (arrow) (haematoxylin and eosin; original magnification $\times 100$).



Figure 7. Immunoreactivity for p27^{Kip1} in epidermal keratinocytes. Fewer nuclei are immunopositive prior to treatment with C225 (a) than 1 week after treatment (b). (Original magnification $\times 40$)

Immunohistochemical findings

The expression of the negative growth regulator p27^{Kip1} was analysed by immunohistochemistry in four patients whose biopsies contained sufficient tissue for analysis. The results are summarized in Table 2. At day 8 of treatment with C225, a three- to fourfold increase in the number of immunolabelled interfollicular epidermal keratinocytes was found. While in normal epidermis only occasional basilar keratinocytes stained positive (Fig. 7a), keratinocytes at all cell layers of the epidermis stained for p27^{Kip1} in skin of treated patients (Fig. 7b).

In situ hybridization

In those cases in which the expression of p27^{Kip1} was analysed by immunohistochemistry, *in situ* hybridization studies were performed in parallel. Labelling was most pronounced in basilar and suprabasilar keratinocytes. There was an approximately twofold increase in cytoplasmic labelling for p27 mRNA in epidermal keratinocytes of treated skin compared with untreated skin (not shown).

Microbiological cultures

Surveillance cultures of facial skin failed to reveal any consistent significant changes in the cutaneous microflora. Neither *Candida* nor bacteria were found on culture at the onset of these lesions, although *S. aureus* was cultured from some persistent lesions.

Discussion

This study describes the cutaneous reactions seen in 10 patients with renal cell carcinoma treated with C225. We focused our analysis on the most common reaction, which was an acneiform follicular eruption, histologically characterized by a lymphocytic perifolliculitis or suppurative superficial folliculitis. The absence of an apparent infectious aetiology, the consistency of the clinical findings across patients, as well as the absence of another drug or plausible aetiology suggest C225 as the causative agent of this follicular dermatitis. Additional indirect evidence for this hypothesis comes from the clinical observation that we have seen a similar acneiform eruption in a patient treated with 4-(3-chloro-4-fluorophenylamino)-7-methoxy-6-(3-(4-morpholinyl) propoxy)-quinazoline, which is known to block EGFR-dependent activation of tyrosine kinase activity.¹⁰

EGFR is expressed in the epidermis, the sweat gland apparatus and hair follicle epithelium.^{11–14} Previous experimental studies have demonstrated a central role of the EGFR in the normal differentiation and development of the hair follicle.^{15–21} Mice harbouring a targeted disruption of the EGFR allele exhibit a disorganized hair follicle phenotype.^{15–21} EGF has pleiotropic effects on keratinocytes. Administration of EGF to newborn mice delays hair follicle development, decreases the rate of growth, and reduces the hair diameter.^{22,23} While it slows the growth and affects the differentiation of hair follicles, EGF is a potent mitogen on cultured keratinocytes and leads to epidermal hyperplasia and hyperkeratosis.^{24–27}

Valuable information on the role of growth factors in the homeostasis of the pilosebaceous unit has been gained from *in vitro* studies.^{28–34} *In vitro* models have demonstrated that EGF and transforming growth factor (TGF)- α are involved in the triggering of anagen and catagen.^{28,29} A critical role for EGF and TGF- α in regulating the differentiation of sebocytes and keratinocytes of the human pilosebaceous infundibulum has been established using organ cultures.^{30,31} EGF and TGF- α , however, are not the only growth factors

relevant for the biology of hair follicles. Several other cytokines, such as insulin-like growth factor, interleukins and tumour necrosis factor- α are also known to play a part.^{32–34}

The effects of C225 on the skin are likely to be a direct consequence of its interference with the signal pathway of EGFR. Among potential candidates of proteins involved in the postreceptor effects of C225, p27^{Kip1} merits attention. It is a negative growth regulator that binds to and inactivates cyclin-dependent kinase-2, thereby leading to cell cycle arrest in G₁.^{35,36} Several *in vitro* studies on various tumour cells have shown that upregulation of p27^{Kip1} plays a crucial part in growth inhibition by C225.^{37–42} Therefore, we examined the effect of C225 treatment on p27^{Kip1} expression in the epidermis by immunohistochemistry and *in situ* hybridization. A three- to fourfold increase in p27^{Kip1}-positive keratinocyte nuclei was observed by the eighth day of treatment, which was accompanied by an increase in p27^{Kip1} mRNA levels. Although it is difficult to draw reliable conclusions from a small number of cases (four patients) in which sufficient lesional tissue was left for analysis, the consistency of increased p27^{Kip1} expression in all cases suggests that that p27^{Kip1} could play a part in mediating the effects of C225 *in vivo*. It also indicates that C225 probably has a direct effect on keratinocytes. Such a direct effect of C225 on target cells was recently demonstrated for mucosal epithelium in a patient with Menetrier's disease.⁴³ Analysis of protein extracts from gastric fundic biopsies suggested that treatment with C225 resulted in lower concentrations of mitogen-associated protein kinase and a decrease in the proliferative index of gastric glandular epithelium.⁴³

C225 probably interferes with the EGFR signal pathway of epidermal and skin adnexal epithelium. The upregulation of p27^{Kip1} observed in our study probably leads to some impairment of cell growth and associated altered differentiation. However, the precise mechanism by which altered levels of p27^{Kip1} contribute to an acneiform eruption remains elusive at the current time. Further experiments are needed to explore the role of p27^{Kip1} in follicular growth and differentiation. It is possible that altered levels of p27^{Kip1} are solely a marker for the effect of C225 and are not causally linked to the acneiform eruption. Other proteins in the signal pathway of EGFR, such as mitogen-associated protein kinase, need to be studied for their role in follicular homeostasis, as they may provide clues to the mechanism of C225 as well as our understanding of the formation of acne.

It is also difficult to establish a causal link between C225 and the presence of an inflammatory reaction. One possible explanation for the inflammatory infiltrate in the biopsies with suppurative inflammation is that it occurs in response to the follicular rupture observed in some specimens. Such a mechanical rupture may have its origin from an effect of C225 on follicular growth and differentiation, which may lead to excessive hyperkeratosis, follicular plugging and subsequent obstruction of the follicular ostium, similar to processes known to occur in acne. A perifollicular inflammatory cell infiltrate in the presence of an intact follicle is more difficult to explain. It is possible that an alteration of follicular growth and differentiation affects the microflora of the skin, especially within the stratum corneum of the follicular infundibulum, which may subsequently elicit an inflammatory reaction. Such a presumed alteration of the cutaneous microflora is not supported by the results from our limited microbiological studies and special stains for micro-organisms. It is, however, conceivable that the inflammatory reaction is more directly related to C225. The mere presence of an antibody on the surface of follicular epithelial cells may directly elicit an inflammatory reaction, which in turn could lead to follicular rupture. Experimental studies are needed to investigate the cause of the inflammation further.

The sparse neutrophilic inflammation involving the terminal portion of the eccrine duct, which was accompanied by focal intraepidermal acantholysis, represents an unusual histological reaction pattern (Fig. 6). We have no explanation for the mechanism of this phenomenon. However, as EGFR is strongly expressed in sweat duct epithelium,¹⁴ alterations in the pathophysiology of eccrine ducts of patients treated with antibodies against EGFR are not a surprise.

The paronychial changes observed in our patients are similar to those previously described with systemic retinoid and antiretroviral therapy.⁴⁴⁻⁴⁶ The specific mechanisms by which these lesions are induced by retinoids and antiretroviral agents are unknown. Our observations suggest a possible role for EGFR in the induction of these lesions.

In summary, we describe the cutaneous side-effects seen in 10 cancer patients treated with C225. We document the histological findings of the most common eruption, which was an acneiform follicular or perifollicular dermatitis. We believe that C225 is the cause of this eruption. To our knowledge, this represents the first report of cutaneous side-effects related to the treatment of patients with a humanized

monoclonal antibody. Our results show that treatment with C225 results in upregulation of p27^{Kip1} in epidermal keratinocytes, which indicates that alteration of p27^{Kip1} may be a mechanism by which C225 can affect follicular and epidermal homeostasis. The similarity of the follicular eruption described herein to acne suggests that further investigations into the disturbance of the EGFR pathway and cell cycle regulation in keratinocytes may be relevant to our understanding of acne and acneiform skin eruptions.

Acknowledgments

The authors thank Kristin Iversen for excellent technical support. This work was supported by a grant from ImClone, New York, NY.

References

- 1 Kawamoto TK, Sato JD, Le AD *et al.* Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc Natl Acad Sci USA* 1983; **80**: 1337-41.
- 2 Sato JD, Kawamoto TK, Le AD *et al.* Biological effects *in vitro* of monoclonal antibodies to human epidermal growth factor receptors. *Mol Biol Med* 1983; **1**: 511-29.
- 3 Prewitt M, Rockwell P, Rockwell RF *et al.* The biologic effects of C225, a chimeric monoclonal antibody to the EGFR, on human prostate carcinoma. *J Immunother* 1996; **19**: 419-27.
- 4 Chou J, Fan Z, Koff A, Mendelsohn J. EGF receptor blockade induces reversible growth arrest and changes in proteins that regulate cell cycle in human epithelial cells. *Proc Am Assoc Cancer Res* 1996; **37**: 10A [Abstr.].
- 5 Mendelsohn J. Epidermal growth factor receptor inhibition by a monoclonal antibody as anticancer therapy. *Clin Cancer Res* 1997; **3**: 2703-7.
- 6 Baselga J, Norton L, Masui H *et al.* Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. *J Natl Cancer Inst* 1993; **85**: 1327-33.
- 7 Bos M, Mendelsohn J, Bowden C *et al.* Phase I studies of anti-epidermal growth factor receptor chimeric monoclonal antibody C225 in patients with EGFR overexpressing tumors. *Am Soc Clin Oncol* 1996; **15**: 443A [Abstr.].
- 8 Cordon-Cardo C, Koff A, Drobnjak M *et al.* Distinct altered patterns of p27Kip1 gene expression in benign prostatic hyperplasia and prostatic carcinoma. *J Natl Cancer Inst* 1998; **90**: 1284-91.
- 9 Park MS, Rosai J, Nguyen HT *et al.* p27 and Rb are on overlapping pathways suppressing tumorigenesis in mice. *Proc Natl Acad Sci USA* 1999; **96**: 6382-7.
- 10 *Investigator's Brochure SD 1839*. Delaware: Zeneca Pharmaceuticals. 1997.
- 11 Green MR, Couchman JR. Distribution of epidermal growth factor receptors in rat tissues during embryonic skin development, hair formation, and the adult hair growth cycle. *J Invest Dermatol* 1984; **83**: 118-23.

- 12 Nanney LB, Magid M, Stoscheck CM, King LE Jr. Comparison of epidermal growth factor binding and receptor distribution in normal human epidermis and epidermal appendages. *J Invest Dermatol* 1984; **83**: 385–93.
- 13 Nanney LB, Stoscheck CM, King LE Jr *et al.* Immunolocalization of epidermal growth factor receptors in normal developing human skin. *J Invest Dermatol* 1990; **94**: 742–8.
- 14 Busam KJ, Tan LK, Granter SR *et al.* Epidermal growth factor, estrogen, and progesterone receptor expression in primary sweat gland carcinomas and primary and metastatic mammary carcinomas. *Mod Pathol* 1999; **12**: 786–93.
- 15 Luetke NC, Qui TH, Pfeiffer RL *et al.* TGF α deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. *Cell* 1993; **73**: 263–78.
- 16 Mann GB, Fowler KJ, Gabriel A *et al.* Mice with a null mutation of the TGF α gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 1993; **73**: 249–61.
- 17 Miettinen PJ, Berger JE, Meneses J *et al.* Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* 1995; **376**: 337–41.
- 18 Murillas R, Larcher F, Contiu CJ *et al.* Expression of a dominant negative mutant of epidermal growth factor receptor in the epidermis of transgenic mice elicits striking alterations in hair follicle development and skin structure. *EMBO J* 1995; **14**: 5216–23.
- 19 Hansen LA, Licht U, Tennenbaum T *et al.* Altered hair follicle morphogenesis in epidermal growth factor receptor deficient mice. In: *Hair Research for the Next Millennium* (van Neste D, Randall VA, eds). Amsterdam: Elsevier Science, 1996: 425–31.
- 20 Hansen LA, Alexander N, Hogan ME *et al.* Genetically null mice reveal a central role for epidermal growth factor receptor in the differentiation of the hair follicle and normal hair development. *Am J Pathol* 1997; **150**: 1959–75.
- 21 Vassar R, Fuchs E. Transgenic mice provide new insights into the role of TGF- α during epidermal development and differentiation. *Genes Dev* 1991; **5**: 714–27.
- 22 Moore GPM, Panaretto BA, Robertson D. Epidermal growth factor delays the development of the epidermis and hair follicles of mice during growth of the first coat. *Anat Rec* 1983; **205**: 47–55.
- 23 Moore GPM, Panaretto BA, Robertson D. Effects of epidermal growth factor on hair growth in the mouse. *J Endocrinol* 1981; **88**: 293–9.
- 24 Cohen S. The stimulation of epidermal proliferation by a specific protein (EGF). *Dev Biol* 1965; **12**: 394–407.
- 25 Moore GPM, Panaretto BA, Carter NB. Epidermal hyperplasia and wool follicle regression in sheep infused with epidermal growth factor. *J Invest Dermatol* 1985; **84**: 172–5.
- 26 Pisansarakit P, Du Cros D, Moore GPM. Cultivation of keratinocytes derived from epidermal explants of sheep skin and the roles of growth factors in the regulation of proliferation. *Arch Dermatol Res* 1990; **281**: 530–5.
- 27 Dominey AM, Wang XJ, King LE Jr *et al.* Targeted overexpression of transforming growth factor α in the epidermis of transgenic mice elicits hyperplasia, hyperkeratosis, and spontaneous squamous papillomas. *Cell Growth Differ* 1993; **4**: 1071–82.
- 28 Philpott MP, Kealey T. Effects of EGF on the morphology and patterns of DNA synthesis in isolated human hair follicles. *J Invest Dermatol* 1994; **102**: 186–91.
- 29 Philpott MP, Sanders DA, Westgate GE, Kealey T. Human hair growth *in vitro*: a model for the study of hair follicle biology. *J Dermatol Sci* 1994; **7**: S55–72.
- 30 Guy R, Green MR, Kealey T. Modeling acne *in vitro*. *J Invest Dermatol* 1996; **106**: 176–82.
- 31 Guy R, Ridden C, Kealey T. The improved organ maintenance of the human sebaceous gland: modeling *in vitro* the effects of epidermal growth factor, androgens, estrogens, 13-*cis* retinoic acid, and phenol red. *J Invest Dermatol* 1996; **106**: 454–60.
- 32 Philpott MP, Sanders DA, Bowen J, Kealey T. Effects of interleukins, colony-stimulating factor and tumour necrosis factor on human hair follicle growth *in vitro*: a possible role for interleukin-1 and tumour necrosis factor- α in alopecia areata. *Br J Dermatol* 1996; **135**: 942–8.
- 33 Rudman SM, Philpott MP, Thomas GA, Kealey T. The role of IGF-I in human skin and its appendages: morphogen as well as mitogen? *J Invest Dermatol* 1997; **109**: 770–7.
- 34 Kealey T, Philpott MP, Guy R. The regulatory biology of the human pilosebaceous unit. *Baillieres Clin Obstet Gynaecol* 1997; **11**: 205–27.
- 35 Polyak K, Lee MH, Erdjument-Bromage H *et al.* Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 1994; **78**: 59–66.
- 36 Polyak K, Kato JY, Solomon MJ *et al.* p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor- β and contact inhibition to cell cycle arrest. *Genes Dev* 1994; **8**: 9–22.
- 37 Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* 1994; **78**: 67–74.
- 38 Wu X, Rubin M, Fan Z *et al.* Involvement of p27KIP1 in G1 arrest mediated by an anti-epidermal growth factor receptor monoclonal antibody. *Oncogene* 1996; **12**: 1397–403.
- 39 Peng D, Fan Z, Lu Y *et al.* Anti-epidermal growth factor receptor monoclonal antibody 225 upregulates p27KIP1 and induces G1 arrest in prostatic cancer cell line DU145. *Cancer Res* 1996; **56**: 3666–9.
- 40 Fan Z, Shang BY, Wu X *et al.* Blockade of epidermal growth factor receptor by anti-epidermal growth factor receptor monoclonal antibody C225 causes G1 arrest of A431 cells with induction of p27KIP1. *Proc Am Assoc Cancer Res* 1996; **17**: 10.
- 41 Fan Z, Shang BY, Lu Y *et al.* Reciprocal changes in p27 (Kip1) and p21 (Cip1) in growth inhibition mediated by blockade or overstimulation of epidermal growth factor receptors. *Clin Cancer Res* 1997; **3**: 1943–8.
- 42 Wang DZM, Hammond VE, Abud HE *et al.* Mutation in Sos1 dominantly enhances a weak allele of the EGFR, demonstrating a requirement for Sos1 in EGFR signaling and development. *Genes Dev* 1997; **11**: 309–20.
- 43 Burdick JS, Chung E, Tanner G *et al.* Treatment of Menetrier's disease with a monoclonal antibody against the epidermal growth factor receptor. *N Engl J Med* 2000; **343**: 1697–701.
- 44 Blumental G. Paronychia and pyogenic granuloma-like lesions with isotretinoin. *J Am Acad Dermatol* 1984; **4**: 677–8.
- 45 Hodak E, David M, Feuerman EJ. Excess granulation tissue during tretinate therapy. *J Am Acad Dermatol* 1984; **11**: 1166–7.
- 46 Tosti A, Piraccini BM, D'Antuono A *et al.* Paronychia associated with antiretroviral therapy. *Br J Dermatol* 1999; **140**: 1165–8.

ImClone Systems Reports Second Quarter and First Six Months 2005 Financial Results

NEW YORK--(BUSINESS WIRE)--July 26, 2005--ImClone Systems Incorporated (NASDAQ: IMCL):

Erbix U.S. In-Market Quarterly Sales Reach \$97.8 Million, Up 12% Over the Prior Quarter, and Up 37% Over the Second Quarter of 2004

Diluted Earnings Per Share of \$.30; Non-GAAP Diluted Earnings Per Share of \$.37 Excluding Expenses Associated with Discontinuance of Small-Molecule Research

ImClone Systems Incorporated (NASDAQ: IMCL) announced today its financial results for the second quarter and six months ended June 30, 2005.

Total revenues for the second quarter of 2005 were \$92.4 million, a 25% increase over the second quarter of 2004. Revenues include four principal components:

License fees and milestone revenue of \$24.5 million in the second quarter of 2005 compared with \$18.1 million in the second quarter of 2004;

Manufacturing revenue of \$7.9 million in the second quarter of 2005 compared with \$14.8 million in the second quarter of 2004. Higher volume purchases by Bristol-Myers Squibb in the second quarter of this year were offset by a lower selling price in 2005 as compared with 2004. Purchases by Bristol-Myers Squibb are timed at their discretion to accommodate forecasts and safety stock needs, and are not necessarily indicative of historical in-market sales or future sales expectations. No product intended for commercial use was sold to Merck KGaA during the second quarter of 2005;

Royalty revenue of \$41.8 million in the second quarter of 2005 compared with \$28.5 million in the second quarter of 2004. Royalty revenue for the second quarter of 2005 includes \$38.1 million representing 39% of Bristol-Myers Squibb's in-market Erbitux net sales of \$97.8 million, compared with first quarter in-market net sales of \$87.1 million, an increase of 12%, and second quarter 2004 in-market net sales of \$71.4 million, an increase of 37%. These in-market sales, reflecting a drop-ship distribution methodology, represent Erbitux shipments to end-user accounts only, with no wholesaler stocking; and

Collaborative agreement revenue of \$18.2 million in the second quarter of 2005 compared with \$12.4 million in the second quarter of 2004. The year-to-year increase principally reflects reimbursement for increased purchases of clinical materials by Merck KGaA, and higher reimbursements for royalty payments.

Total operating expenses for the second quarter of 2005 were \$71.3 million, including \$6.2 million of expenses associated with the discontinuation of the Company's small-molecule research program, which were previously announced on May 11, 2005. Excluding these expenses, operating expenses for the second quarter of 2005 would have been \$65.1 million compared with \$46.4 million in the second quarter of 2004. Operating expenses included:

Research and development expenses for the second quarter of 2005 were \$24.4 million compared with \$18.8 million in the second quarter of 2004; the increase is principally attributable to expenses associated with clinical supplies sold to the Company's partners which are reimbursed as a component of Collaborative agreement revenue;

Clinical and regulatory expenses in the second quarter of 2005 were \$8.4 million, compared with \$6.2 million in the second quarter of 2004, reflecting higher expenses associated with clinical trials in support of Erbitux and pipeline products currently in Phase I clinical development and the preparation of the sBLA for squamous cell carcinoma of the head and neck, which is expected to be filed with the FDA in the third quarter of this year;

Marketing, general and administrative expenses were \$16.8 million in the second quarter of 2005 compared with \$13.6 million in the second quarter of 2004. The increase in 2005 is principally attributable to higher compensation expenses associated with increased headcount, principally in sales and marketing attributable to the field force, and higher professional fees, primarily for legal services;

Royalty expenses were \$14.3 million in the second quarter of 2005 compared with \$7.6 million in the second quarter of 2004. The increase is attributable to higher in-market sales and the fact that the Company did not have an expense as of June 30, 2004 for royalties related to the Centocor license. Approximately \$5.0 million of the 2005 expenses were reimbursed as a component of Collaborative agreement revenue, resulting in net royalty expenses of \$9.3 million for the second quarter of 2005 compared with \$4.4 million in the second quarter of 2004;

Cost of manufacturing revenue was \$1.1 million in the second quarter of 2005 reflecting labeling and packaging costs as well as the recognition of certain previously capitalized manufacturing costs; and

Expenses associated with the discontinuance of the Company's small-molecule research program of \$6.2 million, including \$2.3 million of severance and related costs and \$3.9 million associated with the write-off of fixed assets and other

miscellaneous expenses.

The effective tax rate for the full year of 2005 (assuming no \$250 million milestone is earned from Bristol-Myers Squibb) is now estimated to be 1.0%, principally because of the utilization of deferred tax assets, mainly including the amortization of license fees and milestones which were taxable in prior periods. The Company's estimate of the full-year tax rate at the end of the first quarter of 2005 had been 1.3%; as a result, the effective rate for the second quarter of 2005 is approximately 0.7%.

Net income for the second quarter of 2005 was \$26.0 million compared with \$24.3 million in the second quarter of last year. Diluted earnings per share were \$.30 in the second quarter of 2005 compared with \$.29 in the second quarter of 2004. Excluding the expenses associated with the discontinuation of the Company's small-molecule research program, non-GAAP diluted earnings per share were \$.37 in the second quarter of 2005. A reconciliation of non-GAAP diluted earnings per share to diluted earnings per share prepared in accordance with GAAP is set forth below.

Total revenues and net income for the six months ended June 30, 2005 were \$178.2 million and \$54.9 million, respectively, compared with \$183.9 million and \$87.0 million, respectively, in the first six months of last year. Diluted earnings per share were \$.63, or \$.70 on a non-GAAP basis, for the first six months of 2005 compared with \$1.02 in the first six months of 2004.

Conference Call

ImClone Systems will host a conference call with the financial community to discuss 2005 second quarter and six months financial results, today, July 26, 2005, at 11:00 AM Eastern Daylight Time.

The conference call will be webcast live and may be accessed by visiting ImClone Systems' website at www.imclone.com. A replay of the audio webcast will be available under "Earnings Webcast" in the "Investor Relations" section of the Company's website starting shortly after the call.

Those parties interested in participating via telephone may join by dialing (888) 694-4641, or (973) 935-8512 for calls outside of Canada and the United States. A telephone replay of the conference call will be available shortly after the call until August 2, 2005 at midnight Eastern Daylight Time. To access the telephone replay, dial (877) 519-4471 domestically, or (973) 341-3080 for calls outside of Canada and the United States, and enter passcode number 6272456.

Reconciliation of Diluted Earnings Per Share ("EPS") Excluding Expenses Associated with Discontinuing Small-Molecule Research to Diluted EPS on a GAAP Basis

	Three Months Ended June 30, 2005	Six Months Ended June 30, 2005
EPS excluding expenses associated with discontinuing small-molecule research (1)	\$.37	\$.70
EPS attributable to expenses associated with discontinuing small-molecule research	\$.07	\$.07
EPS, GAAP basis	\$.30	\$.63

(1) EPS excluding expenses associated with discontinuing the Company's small-molecule research program is a non-GAAP financial measure. The Company believes that it is useful to present this non-GAAP financial measure because it permits disclosure of operating results on the same basis used by management and provides investors with a more complete understanding of the Company's underlying operational results and trends. You should not consider EPS excluding expenses associated with discontinuing the Company's small-molecule research program in isolation or as a substitute for EPS determined in accordance with U.S. generally accepted accounting principles, as set forth above.

About ImClone Systems Incorporated

ImClone Systems Incorporated is committed to advancing oncology care by developing and commercializing a portfolio of targeted biologic treatments designed to address the medical needs of patients with a variety of cancers. The Company's research and development programs include growth factor blockers and angiogenesis inhibitors. ImClone Systems' strategy is to become a fully integrated biopharmaceutical company, taking its development programs from the research stage to the

market. ImClone Systems' headquarters and research operations are located in New York City, with additional administration and manufacturing facilities in Branchburg, New Jersey.

Certain matters discussed in this news release may constitute forward-looking statements within the meaning of the Private Securities Litigation Reform Act of 1995 and the Federal securities laws. Although the company believes that the expectations reflected in such forward-looking statements are based upon reasonable assumptions it can give no assurance that its expectations will be achieved. Forward-looking information is subject to certain risks, trends and uncertainties that could cause actual results to differ materially from those projected. Many of these factors are beyond the company's ability to control or predict. Important factors that may cause actual results to differ materially and could impact the company and the statements contained in this news release can be found in the company's filings with the Securities and Exchange Commission including quarterly reports on Form 10-Q, current reports on Form 8-K and annual reports on Form 10-K. For forward-looking statements in this news release, the company claims the protection of the safe harbor for forward-looking statements contained in the Private Securities Litigation Reform Act of 1995. The company assumes no obligation to update or supplement any forward-looking statements whether as a result of new information, future events or otherwise.

IMCLONE SYSTEMS INCORPORATED
Consolidated Condensed Statements of Operations
(Unaudited)
(in thousands, except per share data)

	Three Months Ended June 30,		Six Months Ended June 30,	
	2005	2004 (1)	2005	2004 (1)
Revenues:				
License fees and milestone revenue	\$24,491	\$18,148	\$ 49,025	\$ 85,646
Manufacturing revenue	7,894	14,796	18,913	40,300
Royalty revenue	41,791	28,460	78,163	35,605
Collaborative agreement revenue	18,209	12,364	32,055	22,381
Total revenues	92,385	73,768	178,156	183,932
Operating expenses:				
Research and development	24,433	18,836	45,606	39,047
Clinical and regulatory	8,376	6,202	17,774	13,264
Marketing, general and administrative	16,833	13,577	34,456	25,205
Royalty expense	14,338	7,636	26,904	9,679
Cost of manufacturing revenue	1,106	123	1,849	336
Disposal of small molecule research program	6,200	-	6,200	-
Other	-	-	-	(1,815)
Total operating expenses	71,286	46,374	132,789	85,716
Operating income	21,099	27,394	45,367	98,216
Other (income) expense, net	(5,106)	381	(10,038)	1,496
Income before income taxes	26,205	27,013	55,405	96,720
Provision for income taxes	174	2,701	554	9,672
Net income	\$26,031	\$24,312	\$ 54,851	\$ 87,048

Income per common share:

Basic	\$ 0.31	\$ 0.31	\$ 0.66	\$ 1.14
	=====	=====	=====	=====
Diluted	\$ 0.30	\$ 0.29	\$ 0.63	\$ 1.02
	=====	=====	=====	=====

Shares used in calculation
of income per share:

Basic	83,616	77,373	83,448	76,316
	=====	=====	=====	=====
Diluted	92,074	92,888	92,362	89,620
	=====	=====	=====	=====

- (1) Royalty expense and Collaborative agreement revenue in 2004 have been reclassified to conform to the current year presentation. Both categories have been increased by \$2,231 and \$2,778 for the three and six months ended June 30, 2004, respectively, in order to reflect the reimbursed portion of royalties for agreements that were finalized in January, 2005.

IMCLONE SYSTEMS INCORPORATED
Consolidated Condensed Balance Sheets
(Unaudited)
(in thousands)

Assets	June 30, 2005	December 31, 2004
	-----	-----
Current assets:		
Cash and cash equivalents	\$ 4,588	\$ 79,321
Securities available for sale	807,281	840,451
Inventories	66,412	40,618
Other current assets	88,019	102,047
	-----	-----
Total current assets	966,300	1,062,437
Property, plant and equipment, net	380,950	339,293
Other assets	30,062	33,046
	-----	-----
Total assets	\$ 1,377,312	\$1,434,776
	=====	=====
Liabilities and Stockholders' Equity		
Current liabilities	\$ 226,621	\$ 303,690
Deferred revenue, long term	305,699	348,814
Long-term obligations	602,107	603,434
	-----	-----
Total liabilities	1,134,427	1,255,938
Stockholders' equity	242,885	178,838
	-----	-----
Total liabilities and stockholders' equity	\$ 1,377,312	\$1,434,776
	=====	=====

CONTACT: ImClone Systems Incorporated

Investors:

Andrea F. Rabney, 646-638-5058

Stefania Bethlen, 646-638-5058

or

Media:

David M. F. Pitts, 646-638-5058



PERGAMON

The International Journal of Biochemistry & Cell Biology 31 (1999) 637-643

IJBCB

Molecules in focus EGF receptor

Alan Wells^{a, b, *, 1}^aDepartment of Pathology, University of Alabama, Birmingham, Alabama 35294-0007, USA^bPathology and Laboratory Services, Birmingham VAMC, Birmingham, Alabama 35294-0007, USA

Received 4 December 1998; accepted 2 March 1999

Abstract

The receptor for the epidermal growth factor (EGF) and related ligands (EGFR), the prototypal member of the superfamily of receptors with intrinsic tyrosine kinase activity, is widely expressed on many cell types, including epithelial and mesenchymal lineages. Upon activation by at least five genetically distinct ligands (including EGF, transforming growth factor- α (TGF α) and heparin-binding EGF (HB-EGF)), the intrinsic kinase is activated and EGFR tyrosyl-phosphorylates itself and numerous intermediary effector molecules, including closely-related c-erbB receptor family members. This initiates myriad signaling pathways, some of which attenuate receptor signaling. The integrated biological responses to EGFR signaling are pleiotropic including mitogenesis or apoptosis, enhanced cell motility, protein secretion, and differentiation or dedifferentiation. In addition to being implicated in organ morphogenesis, maintenance and repair, upregulated EGFR signaling has been correlated in a wide variety of tumors with progression to invasion and metastasis. Thus, EGFR and its downstream signaling molecules are targets for therapeutic interventions in wound repair and cancer. Published by Elsevier Science Ltd.

Keywords: Receptor protein tyrosine kinase (RPTK); Tumor invasion; Wound healing; Organogenesis; Signaling pathways

1. Introduction

Cell surface molecules communicate information from the external milieu to the cell. This sensing is critical in multicellular organisms as the cells must function appropriately to their

localization and respond in concert to the needs of the organism. One major family of sensors is comprised of transmembrane receptors with intrinsic protein tyrosine kinase activity (RPTK), the prototypal member of which is the EGF receptor (EGFR; also referred to as HER (human EGF receptor) and c-erbB1) as it was the first receptor described to possess tyrosine kinase activity and the first member of this superfamily to be sequenced. Co-incidentally, the structure of EGFR appears to represent an archetypal pattern for this superfamily of extracellular sensors that control basic cell functions.

* Tel.: 205-934-0357; fax: 205-975-9927.

E-mail address: wells@uab.edu (A. Wells)

¹ I apologize to all authors whose important works contributed to our understanding of the EGF receptor but could not be directly cited in this review due to the limitations in references.

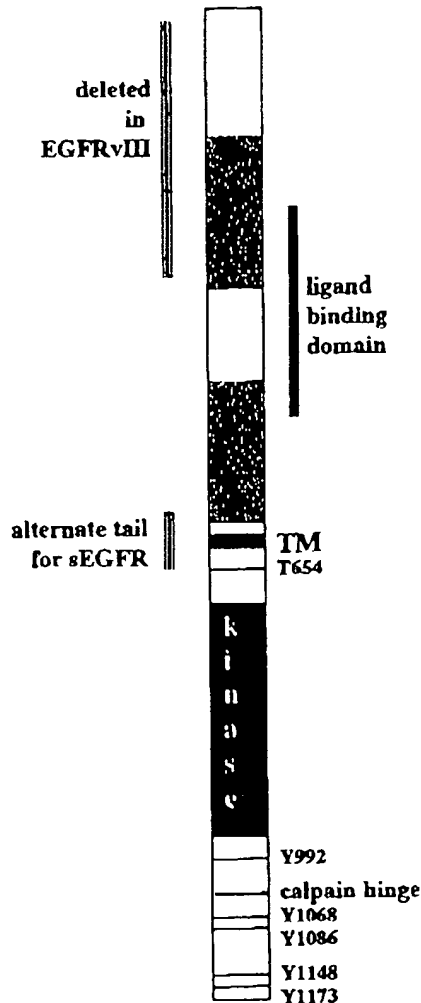


Fig. 1. Structural motifs and regulatory elements in the EGF receptor. The mature EGFR polypeptide is shown. On the left changes which characterize the two variants are shown: the deletion of exons 2-7 in EGFRvIII and the alternatively spliced tail of the secreted EGFR (sEGFR). Other highlighted structures include the two cysteine-rich (CR) domains, the discontinuous ligand-binding domains, which are different but overlapping for the various ligands. The transmembrane (TM) stretch separates the glycosylated extracellular domain from the intracellular regions. This latter includes the tyrosine kinase domain as well as the autophosphorylated tyrosines (Y), the site of PKC transmodulation on threonine at amino acid 654 (T654), and the calpain cleavage site. Not shown are the three internalization domains (at 973, 996, and 1149).

These receptors all present kinase activity directed against tyrosine residues located both within the receptor itself (autophosphorylation) and on target downstream molecules. Ligand binding activates the kinase which, with a possible few minor exceptions, is required for all cellular responses. The pleiotropic cell responses, actuated via still ill-defined pathways, include cell proliferation, migration, and differentiation as well as homeostatic functioning.

2. Structure

EGFR is somewhat unusual among RPTK in that there is a single isoform, from a single 26 exon gene located across 110kb on chromosome 7p11-13, which serves as the sole or overwhelmingly predominant receptor for multiple distinct ligands including EGF, TGF α , amphiregulin, HB-EGF and a number of virally-encoded factors. The protein product of this gene is most often an 1186 amino acid mature transmembrane glycoprotein (Fig. 1). An amino-terminal 622 amino acid extracellular domain containing two cysteine-rich domains comprises the ligand binding domain. There is a single alpha-helical transmembrane pass. The intracellular 542 amino acids can be grouped into three domains. The juxtamembrane domain (~50 amino acids) serves primarily as a site for feedback attenuation by PKC (protein kinase C) and erk MAP kinases (extracellular signal-regulated kinase, mitogen-activated protein kinase), though there is evidence that a motif within this region may link to heterotrimeric G proteins [1]. Next comes a contiguous ~250 amino acid tyrosine kinase (SH1, src homology 1) domain. A unique 229 amino acid long carboxy-terminal tail contains five autophosphorylation motifs which link to proteins containing SH2 or PTB (phospho-tyrosine binding) domains, at least three internalization motifs comprised of a tight turn, and sites for transphosphorylation and proteolytic activation and degradation. This tail also functions as an autoinhibitory substrate; in the absence of either autophosphorylation or removal, ligand-activated EGFR is unable to phosphorylate substrates.

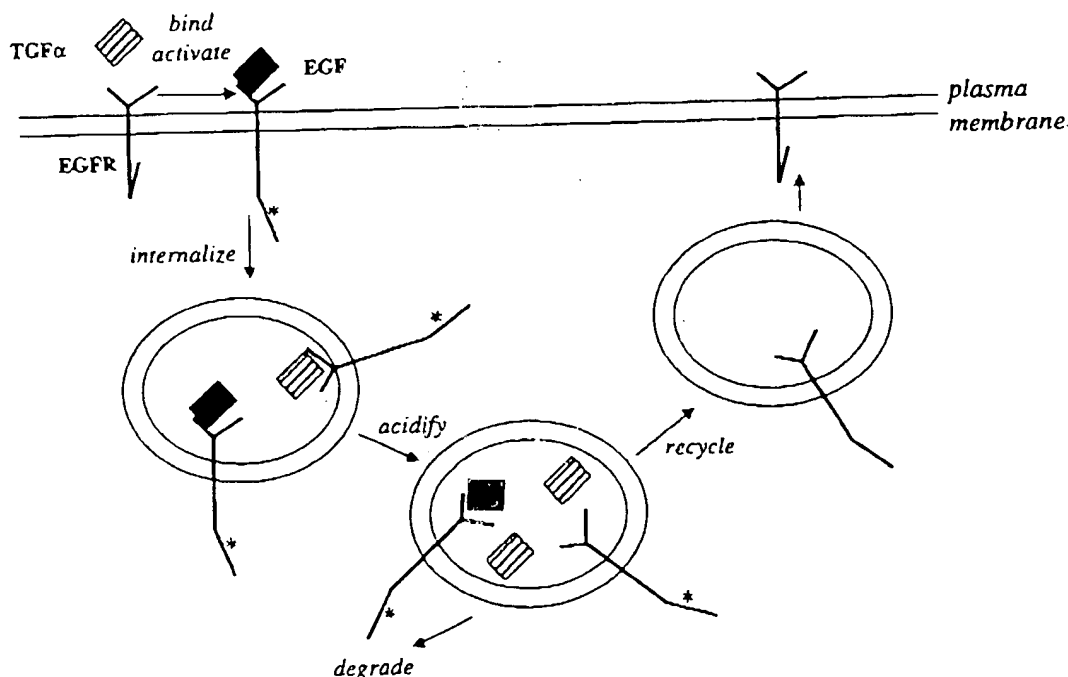


Fig. 2. Ligand-regulated trafficking and disposition of EGFR. Upon ligand binding EGFR kinase is activated and the receptor is internalized via clathrin-coated pits using a saturable, adaptin-specific mechanism. This initial action is consistent between the different ligands. In the acidified endosomal compartment, the ultimate fate of EGFR is determined by the binding properties of the ligand—EGF remains bound and directs the receptor and its bound EGF to degradation; TGFα dissociates and the receptor is recycled while the free TGFα sorts with the majority of the bulk phase to degradation. When this mechanism is saturated (at approximately 50,000 internalized EGFR), excess EGFR is recycled; in this situation EGF bound to receptor will be returned to the surface while the majority of TGFα will still be degraded with the bulk phase.

The EGFR autophosphorylation motifs are structurally similar [2] and functionally redundant, in distinction to many other RPTK. This simple architecture and flexible interchange of redundant motifs bespeaks an archetypal gene.

There are two established and one proposed EGFR variants. A splice variant proximal to the transmembrane domain generates a secreted form of EGFR which can act as a dominant-negative in experimental situations. Whether this acts *in vivo* as a negative titrator of signaling or as a soluble binding protein extending the life-span of EGFR ligands or acting as an extracellular sink for predeposited ligands remains to be determined. A non-ligand binding but constitutively active EGFR variant (EGFRvIII) lacking amino acids 6-273 (exons 2-7) across the first cysteine-

rich domain was first reported as a tumor-specific gene rearrangement; more recent work has suggested that this rearranged gene may replicate a splice variant present during development [3]. The presence of immunologically- and biochemically-defined EGFR in the nucleoplasm has led to suggestions of a transmembrane-negative splice variant similar to one reported for the related *k-sam* gene; however, this species has yet to be positively identified. Except for EGFRvIII in select tumor types, these EGFR variants represent minor populations observed only in limited situations.

EGFR interacts with most members of the *c-erbB* subfamily of RPTK. The ligand for *c-erbB-2* is undefined while *c-erbB-3* and *c-erbB-4* serve as heregulin and neuregulin receptors. However, a

major function of these other receptors appears to be as downstream effectors of each other. These receptors hetero-aggregate, cross-phosphorylate, and modulate signaling from each other in specific pairings. For instance, EGFR (erbB-1) will interact with erbB-2 and erbB-3 but not erbB-4, but erbB-4 will pair with erbB-2. Of particular note is that erbB-3 lacks kinase activity, rather serving as a docking protein to recruit a broader spectrum of downstream effectors after phosphorylation by EGFR or erbB-2. Such a situation may be reiterated by the tumor-specific EGFRvIII.

3. Expression and degradation

EGFR is present on all epithelial and stromal cells as well as select glial and smooth muscle cells. It is transcribed from a TATA-less promoter as two predominant large mRNA species that differ by the extent of 3' untranslated sequence (in humans as ~6 kb and ~9 kb species). Relatively little has been reported about transcriptional control of EGFR, though it appears to decline with cellular aging at least in dermal fibroblasts. Post-translational processing and trafficking has been extensively studied and reviewed. In polarized epithelial cells EGFR is largely restricted to the basolateral aspects, allowing for epithelial-stromal communication from fibroblast-derived TGF α and other matrix-associated EGFR ligands. This asymmetric presentation of EGFR limits autocrine signaling, as many of these epithelial organs, particularly throughout the genitourinary system, secrete copious amounts of EGF into the lumens [4].

Upon ligand binding and activation, EGFR undergo internalization via a saturable endocytic system which depends on specific adaptins and sorting nexins complexing with EGFR carboxy-terminal motifs. The fate of the receptor depends on continued occupancy and kinase activity; EGF, remaining bound in the acidic late endosomal compartment, directs EGFR to degradation whereas activation by TGF α , which displays a pH-sensitive dissociation, results in recycling. Ligand-induced internalization and degradation

results in signal attenuation with net removal of either receptor (in the case of nondissociative ligands like EGF) or ligand (for dissociative ligands such as TGF α) [5] (Fig. 2). Thus, different ligands can dictate the strength and temporal lifespan of EGFR signals, thereby providing a rationale for the existence of multiple genetically-distinct ligands.

4. Biological function

EGFR is a pleiotropic signaler. The integrated biological response to EGFR activation varies from mitogenesis to apoptosis, migration to differentiation to dedifferentiation even in the same cell depending on the context, which includes cell density, type of matrix, other cytokines, and even the position within a cell colony. The molecular bases of these responses is only now being defined [6].

EGFR kinase triggers numerous downstream signaling pathways similar to other RPTK and tyrosine kinase-linked cytokine receptors. These pathways include those that involve PLC γ (phospholipase C- γ) and its downstream calcium- and PKC-mediated cascades, ras activation leading to various MAP kinases, other small GTPases such as rho and rac, multiple STAT (signal transducer and activator of transcription) isoforms, and heterotrimeric G proteins, as well as others to a lesser extent including the phospholipid-directed enzymes PI3 kinase (phosphatidylinositol 3'-OH kinase) and PLD (phospholipase D), and the proto-oncogene cytoplasmic tyrosine kinase src. While this confusion of cascades has prevented lucid exposition of biochemical links to biological responses, a few principles are becoming clear. First, a number of signaling pathways can be shown to be required, but not sufficient for a particular response; PLC γ -mediated hydrolysis of PIP $_2$ (phosphatidylinositol (4,5) biphosphate) and mobilization/activation of actin-modifying proteins is required for EGFR-mediated motility, but motility is blocked if MEK (MAP kinase kinase) signaling is abrogated [6-8]. Second, many signaling pathways contribute to multiple responses; activation of the erk MAP kinases

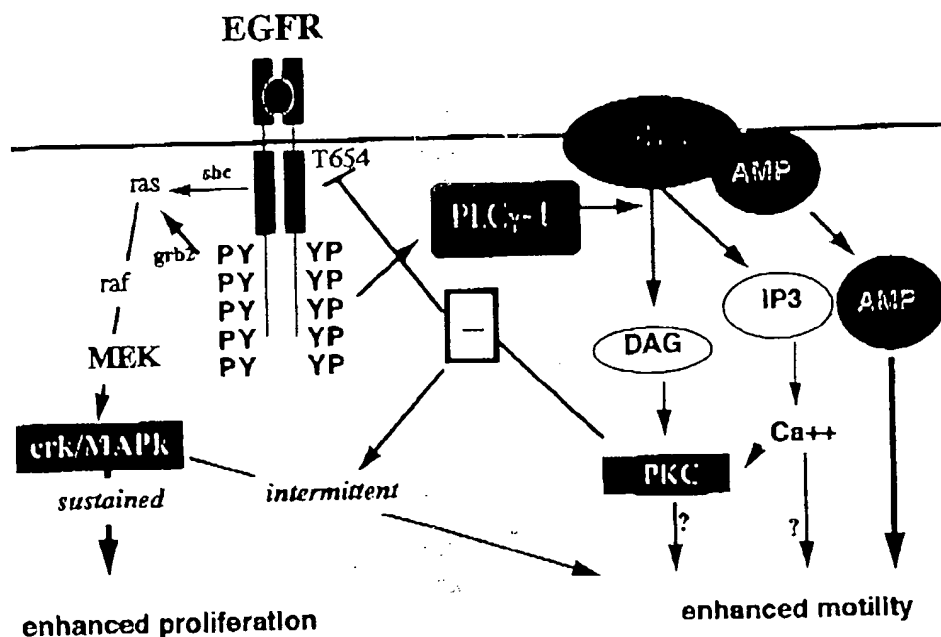


Fig. 3. Crosstalk between downstream pathways activated by EGFR. Ligand binding to EGFR initiates numerous signaling pathways. For many of these pathways, the biological outcomes have yet to be determined. Shown here are the outcomes and interplay of two robustly activated pathways, via PLC γ and ras. The PLC γ pathway not only results in positive signaling for cell motility, by mobilization of actin modifying proteins (AMP) (such as gelsolin, profilin and cofilin) and possibly via protein kinase C (PKC) and calcium-mediated events, but also feedback attenuates EGFR. PKC phosphorylation of EGFR at threonine 654 preferentially disrupts activation of the ras-MAP kinase axis. This is postulated to result in intermittent erk activity which favors motility over mitogenesis.

promotes both proliferation and migration [8]. Third, other signals, both biological and biophysical, modulate the response to EGFR activation; epithelial and stromal cells only exhibit biological responses when attached to a tensed substratum [9]. Fourth, temporal and spatial control of EGFR signaling dictates the biological outcome possibly by altering the balance between various signaling pathways; the motility-associated PLC γ pathway initiates a PKC-mediated feedback attenuation which only slightly decreases global erk activation (but likely alters localized erk populations) which shifts the response from proliferative to migratory [10] (Fig. 3).

All this begs the question of the physiological role of EGFR signaling. This has been approached by extrapolation from in vitro exper-

iments, in vivo perturbations such as disrupting EGFR regulation by adding or blocking ligands, and, most recently, by genetic engineering. The first noted role for EGF was maturation of epithelial tissues, as evinced by precocious eye opening and tooth eruption upon injections of EGF. This developmental role has been supported by EGFR knockouts which die in the neonatal period due to severe immaturity of several epithelial organs; that the pattern and severity of the developmental retardation is dictated by the genetic background of the mice suggests that other RPTK may be recruited to subsume, at least partially, the roles of EGFR. It also was shown that postpartum milk production was enhanced by EGFR signaling, suggesting a metabolic role [11]. In adult animals, EGFR signaling has been postulated as important for organ

repair, which may be viewed as neo-organogenesis; reduction of EGF levels impairs hepatic regeneration. Throughout the genitourinary tract, high levels of luminal EGF are proposed to stimulate repair of epithelial breaks by gaining access to basolateral EGFR.

In most of these situations the operative cellular response is assumed to be the mitogenic response to EGFR signaling. However, during development cells must proliferate, migrate and differentiate, and during repair dedifferentiation may also play a role. Parsing the individual cell responses has been confounded by the pleiotropic nature not only of EGFR signaling but also of several of the downstream effectors. Recently, we have identified PLC γ as being required for EGFR-mediated motility but not mitogenesis. Inhibiting this specific pathway in breast and prostate epithelial cells, severely retards the branching morphogenesis. This pattern is similar to mammary glands in which global EGFR signaling is abrogated by a dominant-negative EGFR [12]. This suggests that the operational response is not mitogenesis but migration. Many further investigations are needed to determine the situations in which each of the integrated cellular responses function physiologically.

5. Medical/industrial application

Control of EGFR signaling will likely provide important opportunities in three main areas—cancer treatment, organ repair, and cell production. EGFR is the receptor most often found upregulated in a wide variety of human tumors [13]. Due to its early identification as the proto-oncogene of the transforming *v-erbB* oncogene and its association with the genesis of numerous tumors, EGFR has been the target of numerous therapies, ranging from therapeutic and imaging antibodies to toxin-linked ligands to enhancement of targeting for gene therapy vectors [14]. These approaches have primarily used upregulated EGFR as a tumor-specific target based on a therapeutic index as EGFR expression is widespread; the special case of EGFRvIII deleted EGFR may, however, represent a 'true' tumor-

specific antigen. In many tumors EGFR levels are not overexpressed but rather signaling is upregulated due to autocrine stimulatory loops secondary to the breakdown of cellular asymmetry and spatial segregation of EGFR and its ligands. In these cancers, the therapeutic index based on EGFR levels is not available. Rather, one needs to evaluate the biology of the tumor. Two cellular responses are considered targets: proliferation and migration. At present, EGFR-mediated but not basal migration can be approached by abrogating activation of PLC γ . We have demonstrated that in a mouse xenograft model of human prostate carcinoma, inhibition of this signaling pathway prevents tumor invasion, albeit tumor growth remains unaffected. Interestingly, this likely represent the pathological aspect of the physiological role of branching morphogenesis. EGFR-specific proliferation, which may be critical for metastatic growth in ectopic sites, currently is less accessible as most downstream targets would likely have widespread unintended toxicities to proliferative organs such as bone marrow and gastrointestinal lining. Still, EGFR-mediated downstream signals may represent a fruitful avenue for attacking tumor invasiveness as an adjunct therapy for anti-mitotic therapy or surgical/radiotherapy bulking.

EGFR modulation holds tremendous promise in promoting wound repair and limiting scarring. One can easily envision that by understanding the quantitative aspects of EGFR signaling on both proliferation and migration, new agents and materials would be developed to improve dermal wound repair, which is readily accessible to repeated and targeted 'drug' applications. During wound repair both proliferation and migration must be triggered but in the proper spatial and temporal context. Our lack of understanding of this complex orchestration likely underlies the failure of EGF and other growth factors to improve this clinical situation to date. A second use bespeaks this fine balance between augmenting and abrogating repair. Injection of high concentration of EGF results in sheep shedding their fleece and has been promoted as an alternative to shearing, though a costlier one. While current human fashion dictates against total hair loss

having a significant cosmetic market, localized depilatory action may be profitable.

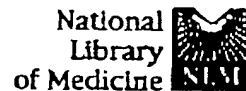
One major industrial use of EGFR signaling is in the production of bio-pharmaceuticals. As genetically-engineered agents are made in mammalian cells, a limiting factor will be control of cell growth and viability. The ready availability and stability of native and recombinant EGFR ligands even in harsh conditions coupled with cross-species promiscuity make these ideal for large-scale cell cultures. Furthermore, specific alterations and modifications have been shown to alter the signaling properties to extend the bioactivity or spare cell receptors [15].

Acknowledgements

Supported by grants from the National Institute of General Medical Sciences (NIH), National Cancer Institute (NIH), Veterans Administration, and the Bioengineering Division of the National Science Foundation. Angela Glading, Jareer Kassis, Jose Souto, and Philip Chang provided important criticism for this review.

References

- [1] H. Sun, Z. Chen, H. Poppleton, K. Scholich, J. Mullenix, G.J. Weipz, D.L. Fulgham, P.J. Berties, T.B. Patel, The juxtamembrane, cytosolic region of the epidermal growth factor receptor is involved in association with α -subunit of G_i, *J. Biol. Chem.* 272 (1997) 5413-5420.
- [2] Z. Songyang, S.E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W.G. Huser, F. King, T. Roberts, S. Ratnofsky, R.J. Lechleider, B.G. Neel, R.B. Birge, J.E. Fajardo, M.M. Chou, H. Hanafusa, B. Schaffhausen, L.C. Cantley, SH2 domains recognize specific phosphopeptide sequences, *Cell* 72 (1993) 767-778.
- [3] R. Nishikawa, X-D. Ji, R. Harmon, C.S. Lazar, G.N. Gill, W.K. Cavenee, H-J.S. Huang, A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity, *Proc. Natl Acad. Sci. (USA)* 91 (1994) 7727-7730.
- [4] U. Marti, S.J. Burwen, A.L. Jones, Biological effects of epidermal growth factor, with emphasis on the gastrointestinal tract and liver: an update, *Hepatology* 9 (1989) 126-138.
- [5] C.C. Reddy, A. Wells, D.A. Lauffenburger, Differential EGF receptor trafficking influences relative mitogenic potencies of epidermal growth factor and transforming growth factor α , *J. Cell Physiol.* 166 (1996) 512-522.
- [6] A. Wells, K. Gupta, P. Chang, S. Swindle, A. Glading, H. Shiraha, Epidermal growth factor receptor-mediated motility in fibroblasts, *Microscopy Research and Techniques* 43 (1998) 395-411.
- [7] P. Chen, J. Murphy-Ullrich, A. Wells, A role for gelsolin in actuating EGF receptor-mediated cell motility, *J. Cell. Biol.* 134 (1996) 689-698.
- [8] H. Xie, M.A. Pallero, D. Gupta, P. Chang, M.F. Ware, W. Witke, D.J. Kwiatkowski, D.A. Lauffenburger, J.E. Murphy-Ullrich, A. Wells, EGF receptor regulation of cell motility: EGF induces disassembly of focal adhesions independently of the motility-associated PLC γ signaling pathway, *J. Cell. Sci.* 111 (1998) 615-624.
- [9] R. Assoian, Anchorage-dependent cell cycle progression, *J. Cell. Biol.* 136 (1997) 1-4.
- [10] P. Chen, H. Xie, A. Wells, Mitogenic signaling from the EGF receptor is attenuated by a motility-associated phospholipase C- γ /protein kinase C feedback mechanism, *Molec. Biol. Cell* 7 (1996) 871-881.
- [11] S. Okamoto, T. Oka, Evidence for physiological function of epidermal growth factor: pregestational sialoadenectomy of mice decreases milk production and increases offspring mortality during lactation period, *Proc. Natl Acad. Sci. (USA)* 81 (1984) 6059-6063.
- [12] W. Xie, A.J. Paterson, E. Chin, L.M. Nabell, J.E. Kudlow, Targeted expression of a dominant negative epidermal growth factor receptor in the mammary gland of transgenic mice inhibits pubertal mammary duct development, *Molec. Endocrinol.* 11 (1998) 1766-1781.
- [13] S.A. Aaronson, Growth factors and cancer, *Science* 254 (1991) 1146-1153.
- [14] B.R. Voldborg, L. Damstrup, M. Spang-Thomsen, H.S. Poulsen, Epidermal growth factor receptor (EGFR) and EGFR mutations, functions and possible role in clinical trials, *Ann. Oncology* 8 (1997) 1197-1206.
- [15] C.C. Reddy, S.K. Niyogi, A. Wells, H.S. Wiley, D.A. Lauffenburger, Re-engineering epidermal growth factor for enhanced potency, *Nature Biotechnol.* 14 (1996) 1696-1699.



PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM

Search PubMed

for

Limits

Preview/Index

History

Clipboard

Details

About Entrez

Display Abstract Show: 20 Sort Text

Text Version

1: Dan Med Bull 1998 Apr;45(2):121-34

Related Articles, Links

Entrez PubMed

Overview

Help | FAQ

Tutorial

New/Noteworthy

E-Utilities

PubMed Services

Journals Database

MeSH Browser

Single Citation Matcher

Batch Citation Matcher

Clinical Queries

LinkOut

Cubby

Related Resources

Order Documents

NLM Gateway

TOXNET

Consumer Health

Clinical Alerts

ClinicalTrials.gov

PubMed Central

Privacy Policy

The EGF receptor system in head and neck carcinomas and normal tissues. Immunohistochemical and quantitative studies.

Christensen ME.

Department of Otolaryngology-Head and Neck Surgery, H:S Rigshospitalet, Copenhagen.

The EGF receptor (EGF receptor) and two of the ligands, transforming growth factor alpha (TGF-alpha) and epidermal growth factor (EGF), exert mitogenic activities in epithelial cells. Hence, the overall aim of this work was delineation of the EGF receptor system in head and neck carcinomas, which in the majority of cases are epithelial derived tumours. Chapter 1 is a general introduction to head and neck carcinomas and the relevance of the EGF receptor system in this context. Chapter 2 focuses on the immunohistochemical distribution of TGF-alpha in normal human tissues, while previous studies dealing with the growth factor in head and neck carcinomas revealed other localizations in normal cells. TGF-alpha was detected with monoclonal as well as polyclonal antibodies. The results showed that the growth factor is widely distributed in normal human tissues and thus not limited to malignant cells. Chapter 3 describes the immunohistochemical localization of the EGF receptor in 55 patients with squamous cell carcinoma in the head and neck region. The study included adjacent normal mucosa and in 12 cases additional dysplastic areas were present. The EGF receptor was found in the basal cell layer in normal oral and laryngeal mucosa. In sections from patients who had received preoperative irradiation the receptor was in addition seen on the spinous cells. In dysplastic epithelial all cells stained for the EGF receptor. The majority of the head and neck carcinomas expressed the EGF receptor. In poorly differentiated tumours almost all cells were positive for the receptor. Sections from moderately and well differentiated tumours demonstrated a reduction in the extent of stained areas, paralleling the situation observed in the differentiated upper layers of normal oral and laryngeal mucosa. Furthermore, this chapter describes the EGF receptor quantitatively in 60 patients with head and neck carcinoma. This study was performed in order

to evaluate if overexpression of the EGF receptor was a common motif for head and neck carcinomas. The level in tumour biopsies was compared with the level in the patients' corresponding normal mucosa. An enzyme-linked immunosorbent assay detecting protein epitopes of the receptor was employed. Overexpression of the receptor was found in the majority of cases. The overexpression was further correlated to clinicopathological parameters. However, no significant correlations were found although the mean values increased with increased tumour size and advanced clinical stage. The use of quantitative assays are further discussed and limitations are emphasized with respect to heterogeneity at the EGF receptor level and the varying stromal components in malignant tissues. Despite these problems the relevance of the EGF receptor a therapeutic situation is illustrated with e.g. EGF receptor antibodies and tyrosine-kinase inhibitors. Chapter 4 focuses on the immunohistochemical expression of EGF and TGF-alpha in carcinomas from same 55 patients. This study included adjacent normal mucosa in which the growth factors were expressed above the basal cell layer. The majority of the tumours expressed both growth factors and none of the sections were negative for both EGF and TGF-alpha. In biopsies from moderately and well differentiated tumours the growth factors were demonstrated in the more differentiated cells. However, in poorly differentiated tumours the cells were positive for EGF and TGF-alpha. Chapter 5 describes immunohistochemical and quantitative changes of salivary EGF, amylase and haptocorrin following radiotherapy for oral cancer. This study was initiated because irradiated oral and laryngeal mucosa have demonstrated staining for the receptor in the basal cell layer as well as in the spinous cells, indicating an upregulation of the receptor in response to lack of EGF. In normal biopsies from the glandula submandibularis and glandula parotis, EGF and amylase were demonstrated in the serous acini, whereas haptoc

Publication Types:

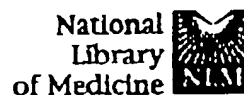
- Review
- Review, Academic

PMID: 9587699 [PubMed - indexed for MEDLINE]

[Display](#) [Abstract](#) [Show](#) [20](#) [Sort](#) [Send to](#) [Text](#)

[Write to the Help Desk](#)
[NCBI](#) | [NLM](#) | [NIH](#)
[Department of Health & Human Services](#)
[Freedom of Information Act](#) | [Disclaimer](#)

Mar 17 2003 10:44:0



[PubMed](#)
[Nucleotide](#)
[Protein](#)
[Genome](#)
[Structure](#)
[PMC](#)
[Taxonomy](#)
[OMIM](#)

Search for

[Limits](#)
[Preview/Index](#)
[History](#)
[Clipboard](#)
[Details](#)

[About Entrez](#)

☐ Abstract
 Show:

Text Version

[Entrez PubMed](#)
[Overview](#)
[Help | FAQ](#)
[Tutorial](#)
[New/Noteworthy](#)
[E-Utilities](#)

[PubMed Services](#)
[Journals Database](#)
[MeSH Browser](#)
[Single Citation Matcher](#)
[Batch Citation Matcher](#)
[Clinical Queries](#)
[LinkOut](#)
[Cubby](#)

[Related Resources](#)
[Order Documents](#)
[NLM Gateway](#)
[TOXNET](#)
[Consumer Health](#)
[Clinical Alerts](#)
[ClinicalTrials.gov](#)
[PubMed Central](#)

[Privacy Policy](#)

1: Prog Neurobiol 1996 Jun;49(2):99-123

[Related Articles](#) [Links](#)

[ELSEVIER SCIENCE](#)
[FULL-TEXT ARTICLE](#)

Transforming growth factor-alpha (TGF-alpha) and epidermal growth factor-receptor (EGF-R) immunoreactivity in normal and pathologic brain.

Ferrer I, Alcantara S, Ballabriga J, Olive M, Blanco R, Rivera R, Carmona M, Berruezo M, Pitarch S, Planas AM.

Unitat de Neuropatologia, Hospital Princeps d'Espanya, Universitat de Barcelona, Spain.

Transforming growth factor alpha (TGF-alpha) and epidermal growth factor-receptor (EGF-R) immunoreactivity is observed in the majority of neurons, and in maturing astrocytes, in the developing and adult brain of humans and different species of animals. TGF-alpha and EGF-R co-localize in most neurons and maturing astrocytes, suggesting that most TGF-alpha-producing cells are EGF-R-expressing cells. TGF-alpha and EGF-R immunoreactivity decrease in damaged areas following different insults. However, EGF-R appears in reactive glia, mostly reactive astrocytes, within and surrounding the damaged areas. TGF-alpha and EGF-R immunoreactivity is found in neurons of patients affected by Alzheimer's disease and other forms of dementia, and in neurons of patients suffering from epilepsy owing to different causes, thus pointing to the conclusion that TGF-alpha does not play a significant role in these pathologies. However, EGF-R immunoreactivity occurs in reactive astrocytes and microglia in subacute but not chronic lesions in human cases. Since TGF-alpha is a membrane-anchored growth factor, which may be cleaved leading to the formation of soluble forms, and both the membrane-anchored and soluble forms have the capacity to activate the EGF-R, it is feasible that TGF-alpha in the nervous system may act upon EGF-R-containing neurons through different mechanisms. In addition to distant effects resulting from the release of soluble TGF-alpha, local effects may be produced by establishing direct cell-to-cell contacts (juxtacrine stimulation), or in cells expressing both TGF-alpha and EGF-R (autocrine stimulation).

Monoclonal Antibody 806 Inhibits the Growth of Tumor Xenografts Expressing Either the de2-7 or Amplified Epidermal Growth Factor Receptor (EGFR) but not Wild-Type EGFR

Rodney B. Luwor, Terrance G. Johns,¹ Carmel Murone, H-J. Su Huang, Webster K. Cavenee, Gerd Ritter, Lloyd J. Old, Antony W. Burgess, and Andrew M. Scott

Ludwig Institute for Cancer Research, Melbourne Branch, Tumour Targeting Program, Austin and Repatriation Medical Centre, Heidelberg 3084, Victoria, Australia [R. B. L., T. G. J., C. M., A. M. S.]; Epithelial Biochemistry Laboratory, Royal Melbourne Hospital, Parkville 3050 Victoria, Australia [A. W. B.]; Ludwig Institute for Cancer Research, San Diego Branch, University of California at San Diego, La Jolla, California 92093-0660 [H.-J. S. H., W. K. C.]; and Ludwig Institute for Cancer Research, New York Branch, New York, New York 10021-6007 [G. R., L. J. O.]

Abstract

The monoclonal antibody (mAb) 806 was raised against the delta2-7 epidermal growth factor receptor (de2-7 EGFR or EGFRvIII), a truncated version of the EGFR commonly expressed in glioma. Unexpectedly, mAb 806 also bound the EGFR expressed by cells exhibiting amplification of the *EGFR* gene but not to cells or normal tissue expressing the wild-type receptor in the absence of gene amplification. The unique specificity of mAb 806 offers an advantage over current EGFR antibodies, which all display significant binding to the liver and skin in humans. Therefore, we examined the antitumor activity of mAb 806 against human tumor xenografts grown in nude mice. The growth of U87 MG xenografts, a glioma cell line that endogenously expresses $\sim 10^5$ EGFRs in the absence of gene amplification, was not inhibited by mAb 806. In contrast, mAb 806 significantly inhibited the growth of U87 MG xenografts transfected with the de2-7 EGFR in a dose-dependent manner using both preventative and established tumor models. Significantly, U87 MG cells transfected with the wild-type EGFR, which increased expression to $\sim 10^6$ EGFRs/cell and mimics the situation of gene amplification, were also inhibited by mAb 806 when grown as xenografts in nude mice. Xenografts treated with mAb 806 all displayed large areas of necrosis that were absent in control tumors. This reduced xenograft viability was not mediated by receptor down-regulation or clonal selection because levels of antigen expression were similar in control and treated groups. The antitumor effect of mAb 806 was not restricted to U87 MG cells because the antibody inhibited the growth of new and established A431 xenografts, a cell line expressing $>10^6$ EGFRs/cell. This study demonstrates that mAb 806 possesses significant antitumor activity.

Introduction

Over the past two decades, mAbs² have attracted considerable interest as potential agents for the treatment of human cancer (1, 2). A number of these mAbs have been directed to the EGFR, which given its increased expression on the cell surface of many human tumors is a candidate for antibody therapy. Overexpression of the EGFR has been observed in tumors of the breast, lung, colon, prostate, kidney, bladder, head and neck, ovary, and brain (3, 4), with increased EGFR expression levels often correlating with a poorer clinical prognosis (5, 6). Overexpression of the EGFR can be associated with *EGFR* gene amplification, particularly in glioma and head and neck cancer.

Some of the current EGFR-specific mAbs are capable of inhibiting the *in vitro* and *in vivo* growth of epithelial tumor cells overexpressing the EGFR by blocking ligand binding (7-10). Although several EGFR mAbs have been evaluated for tumor-specific targeting and pharmacokinetics in clinical trials (11, 12), their use is restricted by specific uptake in organs that have high endogenous levels of EGFR, such as the liver and skin (11, 13). For example, it would not be possible to conjugate such antibodies to cytotoxic agents for the purposes of therapy because this would almost certainly cause significant collateral damage to normal tissue.

EGFR gene amplification in glioma is often accompanied by gene rearrangement, resulting in deletions of the coding region (14). The most common variant, the de2-7 EGFR, is characterized by an in-frame deletion of 801 bp spanning exons 2-7 of the coding sequence (15). This truncation removes 267 amino acids from the extracellular domain, producing a unique junctional peptide, and renders the EGFR unable to bind any known ligand (4). Despite this, the truncation appears to partially mimic the effect of ligand binding because the receptor displays low levels of constitutive activation (16). Furthermore, glioma and breast cells transfected with the de2-7 EGFR have enhanced tumorigenicity when grown as xenografts in nude mice (16, 17). Apart from glioma, the de2-7 EGFR has been identified in breast, non-small cell lung, ovarian, and prostate cancer (3, 18, 19) but has not been found in normal tissue (20). Therefore, targeting this tumor-specific antigen may permit broader therapeutic strategies than is possible using wt EGFR-based immunotherapeutic strategies. Indeed, several mAbs specific for the unique junctional peptide found in the de2-7 EGFR have been described (20-22).

The de2-7 EGFR specific mAb 806 was produced after immunization of mice with NR6 mouse fibroblasts expressing the truncated de2-7 EGFR. mAb 806 binds the U87 MG glioma cell line transfected with the de2-7 EGFR but not the parental U87 MG cell line, which expresses the wt EGFR without gene amplification.³ Similar results were observed *in vivo* with mAb 806 showing specific targeting of de2-7 EGFR expressing U87 MG xenografts but not parental U87 MG tumors.³ Interestingly, mAb 806 was capable of binding an EGFR subset ($\sim 10\%$) on the surface of the A431 cell line, which contains an amplified *EGFR* gene. Therefore, unlike all other de2-7 EGFR-specific antibodies, which recognize the unique peptide junction that is generated by the de2-7 EGFR truncation, mAb 806 binds to an epitope also found in overexpressed wt EGFR. However, it

Received 4/2/01; accepted 5/30/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ To whom requests for reprints should be addressed, at Tumour Targeting Program, Ludwig Institute for Cancer Research, Austin and Repatriation Medical Centre, Studley Road, Heidelberg 3084, Victoria, Australia. Phone: 613-9496-3068; Fax: 613-9496-5892; E-mail: Terry.Johns@ludwig.edu.au.

² The abbreviations used are: mAb, monoclonal antibody; de2-7, delta2-7; EGFR, epidermal growth factor receptor; wt, wild type; FACS, fluorescence-activated cell sorter; HSA, human serum albumin.

³ T. G. Johns, E. Stockert, G. Ritter, A. A. Jungbluth, H.-J. S. Huang, W. K. Cavenee, F. E. Smyth, C. M. Hall, N. Watson, E. C. Nice, L. J. Old, A. W. Burgess, and A. M. Scott. A novel monoclonal antibody specific for the de2-7 epidermal growth factor receptor (EGFR) that also recognizes the EGFR expressed in cells containing amplification of the *EGFR* gene, submitted for publication.

would appear that this epitope is preferentially exposed in the de2-7 EGFR and a small proportion of receptors expressed in cells containing wt *EGFR* gene amplification. Importantly, normal tissues that express high levels of endogenous wt EGFR, such as liver and skin, are negative for mAb 806 binding. On the basis of the unique property of the mAb 806 to bind both the de2-7 and amplified wt EGFR but not the native wt EGFR when expressed at normal levels, we decided to examine the efficacy of mAb 806 against several tumor cell lines grown as xenografts in nude mice.

Materials and Methods

Cell Lines and Monoclonal Antibodies. The human glioblastoma cell line U87 MG, which endogenously expresses the wt EGFR, and the transfected cell lines U87 MG.Δ2-7 and U87 MG.wtEGFR, which express the de2-7 EGFR and overexpress the wt EGFR, respectively, have been described previously (16, 23). The epidermoid carcinoma cell line A431 has been described previously (24).

All cell lines were maintained in DMEM (DMEM/F12; Life Technologies, Inc., Grand Island, NY) containing 10% FCS (CSL, Melbourne, Victoria, Australia), 2 mM glutamine (Sigma Chemical Co., St. Louis, MO), and penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY). In addition, the U87 MG.Δ2-7 and U87 MG.wtEGFR cell lines were maintained in 400 μg/ml of genetin (Life Technologies, Inc., Melbourne, Victoria, Australia). Cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂.

The mAb 806 (IgG2b) was produced after immunization of mice with NR6 mouse fibroblasts expressing the de2-7 EGFR. mAb 806 was selected after rosette assays showed binding to NR6 cells, which overexpressed the de2-7 EGFR (titer of 1:2500). mAb 528, which recognizes both de2-7 and wt EGFR, has been described previously (10) and was produced in the Biological Production Facility (Ludwig Institute for Cancer Research, Melbourne, Victoria, Australia) using a hybridoma obtained from American Type Culture Collection (Rockville, MD). The DH8.3 mAb, which is specific for the de2-7 EGFR, was kindly provided by Prof. William Gullick (University of Kent and Canterbury, Kent, United Kingdom) (19). The polyclonal antibody sc-03 directed to the COOH-terminal domain of the EGFR was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA).

FACS Analysis of Receptor Expression. Cultured parental and transfected U87 MG cell lines were analyzed for wt and de2-7 EGFR expression using the 528, 806, and DH8.3 antibodies. Cells (1×10^6) were incubated with 5 μg/ml of the appropriate antibody or an isotype-matched negative control in PBS containing 1% HSA for 30 min at 4°C. After three washes with PBS/1% HSA, cells were incubated an additional 30 min at 4°C with FITC-coupled goat antimouse antibody (1:100 dilution; Calbiochem, San Diego, CA). After three subsequent washes, cells were analyzed on an Epics Elite ESP (Beckman Coulter, Hialeah, FL) by observing a minimum of 20,000 events and analyzed using EXPO (version 2) for Windows.

Scatchard Analysis. The mAb 806 was labeled with ¹²⁵I (Amrad, Melbourne, Victoria, Australia) by the Chloramine T method. All binding assays were performed in 1% HSA/PBS on $1-2 \times 10^6$ live U87 MG.Δ2-7 or A431 cells for 90 min at 4°C with gentle rotation. A set concentration of 10 ng/ml ¹²⁵I-labeled mAb 806 was used in the presence of increasing concentrations of unlabeled antibody. Nonspecific binding was determined in the presence of 10,000-fold excess of unlabeled antibody. After incubation, cells were washed and counted for bound ¹²⁵I-labeled mAb 806 using a COBRA II gamma counter (Packard Instrument Company, Meriden, CT). Scatchard analysis was done after correction for immunoreactivity.

Immunoprecipitation Studies. Cells were labeled for 16 h with 100 μCi/ml of Tran³⁵S-Label (ICN Biomedicals, Irvine, CA) in DMEM without methionine/cysteine supplemented with 5% dialyzed FCS. After washing with PBS, cells were placed in lysis buffer (1% Triton X-100, 30 mM HEPES, 150 mM NaCl, 500 μM 4-(2-aminoethyl) benzenesulfonyl fluoride, 150 mM aprotinin, 1 μM E-64 protease inhibitor, 0.5 mM EDTA, and 1 μM leupeptin, pH 7.4) for 1 h at 4°C. Lysates were clarified by centrifugation for 10 min at 12,000 × g and then incubated with 5 μg of appropriate antibody for 30 min at 4°C before the addition of protein A-Sepharose. Immunoprecipitates were washed three times with lysis buffer, mixed with SDS sample buffer, separated

by gel electrophoresis using a 7.5% gel that was then dried, and exposed to X-ray film.

Xenograft Models. Consistent with previous reports (23, 25), U87 MG cells transfected with de2-7 EGFR grew more rapidly than parental cells and U87 MG cells transfected with the wt EGFR. Tumor cells (3×10^6) in 100 μl of PBS were inoculated s.c. into both flanks of 4-6-week-old, female nude mice (Animal Research Center, Western Australia, Perth, Australia). Therapeutic efficacy of mAb 806 was investigated in both preventative and established tumor models. In the preventative model, five mice with two xenografts each were treated i.p. with either 0.1 or 1 mg of mAb 806 or vehicle (PBS) starting the day before tumor cell inoculation. Treatment was continued for a total of six doses, three times per week for 2 weeks. In the established model, treatment was started when tumors had reached a mean volume of 65 mm³ (U87 MG.Δ2-7), 84 mm³ (U87 MG), 73 mm³ (U87 MG.wtEGFR), or 201 mm³ (A431 tumors). Tumor volume in mm³ was determined using the formula (length × width²)/2, where length was the longest axis and width the measurement at right angles to the length (26). Data were expressed as mean tumor volume ± SE for each treatment group. This research project was approved by the Animal Ethics Committee of the Austin and Repatriation Medical Centre.

Histological Examination of Tumor Xenografts. Xenografts were excised at the times indicated and bisected. One half was fixed in 10% formalin/PBS before being embedded in paraffin. Four-μm sections were then cut and stained with H&E for routine histological examination. The other half was embedded in Tissue Tek OCT compound (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen, and stored at -80°C. Thin (5-μm) cryostat sections were cut and fixed in ice-cold acetone for 10 min, followed by air drying for an additional 10 min. Sections were blocked in protein blocking reagent (Lipshaw Immunon, Pittsburgh, PA) for 10 min and then incubated with biotinylated primary antibody (1 μg/ml) for 30 min at room temperature. All antibodies were biotinylated using the ECL protein biotinylation module (Amersham, Baulkham Hills, NSW, Australia), as per the manufacturer's instructions. After rinsing with PBS, sections were incubated with a streptavidin-horseradish peroxidase complex for an additional 30 min (Silenus, Melbourne, Victoria, Australia). After a final PBS wash, the sections were exposed to 3-amino-9-ethylcarbazole substrate [0.1 M acetic acid, 0.1 M sodium acetate, 0.02 M 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO)] in the presence of hydrogen peroxide for 30 min. Sections were rinsed with water and counterstained with hematoxylin for 5 min and mounted.

Statistical Analysis. The *in vivo* tumor measurements in mm³ are expressed as the mean ± SE. Differences between treatment groups at given time points were tested for statistical significance using Student's *t* test.

Results

Binding of Antibodies to Cell Lines. To determine the specificity of mAb 806, its binding to U87 MG, U87 MG.Δ2-7, and U87 MG.wtEGFR cells was analyzed by FACS. An irrelevant IgG2b (mAb 100-310 directed to the human antigen A33) was included as an isotype control for mAb 806, and the 528 antibody was included because it recognizes both the de2-7 and wt EGFR. Only the 528 antibody was able to stain the parental U87 MG cell line (Fig. 1), consistent with previous reports demonstrating that these cells express the wt EGFR (16). mAb 806 had binding levels similar to the control antibody, clearly demonstrating that it is unable to bind the wt EGFR (Fig. 1). Binding of the isotype control antibody to the U87 MG.Δ2-7 and U87 MG.wtEGFR cell lines was similar to that observed for the U87 MG cells. mAb 806 stained U87 MG.Δ2-7 and U87 MG.wtEGFR cells, indicating that mAb 806 specifically recognized the de2-7 EGFR and a subset of the overexpressed EGFR (Fig. 1). As expected, the 528 antibody stained both the U87 MG.Δ2-7 and U87 MG.wtEGFR cell lines (Fig. 1). The intensity of 528 antibody staining on U87 MG.wtEGFR cells was much higher than mAb 806, suggesting that mAb 806 only recognizes a portion of the overexpressed EGFR. The mAb 806 reactivity observed with U87 MG.wtEGFR cells is similar to that obtained with A431 cells, another cell line that overexpresses the wt EGFR.³

A Scatchard analysis was performed using U87 MG.Δ2-7 and

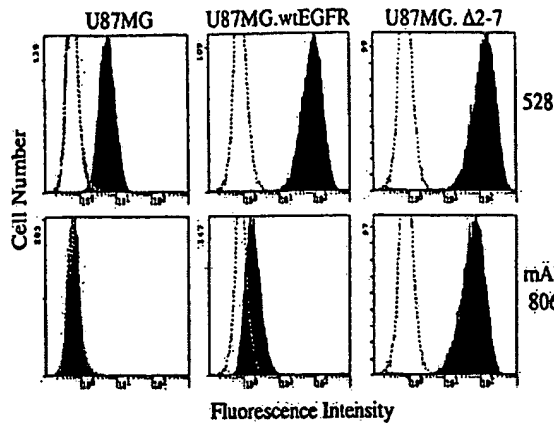


Fig. 1. Flow cytometric analysis of parental and transfected U87 MG glioma cell lines. Cells were stained with either an irrelevant IgG2b antibody (open histograms) or the 528 antibody or mAb 806 (filled histograms) as indicated.

A431 cells to determine the relative affinity and binding sites for mAb 806 on each cell line. mAb 806 had an affinity for the $\Delta 2-7$ EGFR receptor of $1.1 \times 10^9 \text{ M}^{-1}$ and recognized an average (three separate experiments) of 2.4×10^5 binding sites/cell. In contrast, the affinity of mAb 806 for the wt EGFR on A431 cells was only $9.5 \times 10^7 \text{ M}^{-1}$. Interestingly, mAb 806 recognized 2.3×10^5 binding sites on the surface of A431, which is some 10-fold lower than the reported number of EGFR found in these cells. To confirm the number of EGFR on the surface of our A431 cells, we performed a Scatchard analysis using ^{125}I -labeled 528 antibody. As expected, this antibody bound to approximately 2×10^6 sites on the surface of A431 cells. Thus, it appears that mAb 806 only binds a portion of the EGFR receptors on the surface of A431 cells. Importantly, ^{125}I -labeled mAb 806 did not bind to the parental U87 MG cells at all, even when the number of cells was increased to 1×10^7 .

Immunoprecipitations. We further characterized mAb 806 reactivity in the various cell lines by immunoprecipitation after ^{35}S -labeling using mAb 806, sc-03 (a commercial polyclonal antibody specific for the COOH-terminal domain of the EGFR) and a IgG2b isotype control. The sc-03 antibody immunoprecipitated three bands from U87 MG.Δ2-7 cells, a doublet corresponding to the two $\Delta 2-7$ EGFR bands observed in these cells and a higher molecular weight band corresponding to the wt EGFR (Fig. 2). In contrast, although mAb 806 immunoprecipitated the two $\Delta 2-7$ EGFR bands, the wt EGFR was completely absent (Fig. 2). The pattern seen in U87 MG.wtEGFR and A431 cells was essentially identical. The sc-03 antibody immunoprecipitated a single band corresponding to the wt EGFR from both cell lines (Fig. 2). The mAb 806 also immunoprecipitated a single band corresponding to the wt EGFR from both U87 MG.wtEGFR and A431 cells (Fig. 2). Consistent with the FACS and Scatchard data, the amount of EGFR immunoprecipitated by mAb 806 was substantially less than the total EGFR present on the cell surface. Given that mAb 806 and the sc-03 immunoprecipitated similar amounts of the $\Delta 2-7$ EGFR, this result supports the notion that the mAb 806 antibody only recognizes a portion of the EGFR in cells overexpressing the receptor. Comparisons between mAb 806 and the 528 antibody showed an identical pattern of reactivity (data not shown). An irrelevant IgG2b (an isotype control for mAb 806) did not immunoprecipitate EGFR from any of the cell lines (Fig. 2). Using identical conditions, mAb 806 did not immunoprecipitate the EGFR from the parental U87 MG cells (data not shown).

Efficacy of mAb 806 in Preventative Models. mAb 806 was examined for efficacy against U87 MG and U87 MG.Δ2-7 tumors in

a preventative xenograft model. Antibody or vehicle was administered i.p. the day before tumor inoculation and was given three times per week for 2 weeks (see "Materials and Methods"). At a dose of 1 mg/injection, mAb 806 had no effect on the growth of parental U87 MG xenografts that express the wt EGFR (Fig. 3A). In contrast, mAb 806 inhibited significantly the growth of U87 MG.Δ2-7 xenografts in a dose-dependent manner (Fig. 3B). Twenty days after tumor inoculation, when control animals were sacrificed, the mean tumor volume was $1600 \pm 180 \text{ mm}^3$ for the control group, a significantly smaller $500 \pm 95 \text{ mm}^3$ for the 0.1 mg/injection group ($P < 0.0001$) and $200 \pm 42 \text{ mm}^3$ for the 1 mg/injection group ($P < 0.0001$). Treatment groups were sacrificed at day 24, at which time the mean tumor volumes were $1300 \pm 240 \text{ mm}^3$ for the 0.1 mg treated group and $500 \pm 100 \text{ mm}^3$ for the 1 mg group ($P < 0.005$).

Efficacy of mAb 806 in Established Xenograft Models. Given the efficacy of mAb 806 in the preventative xenograft model, its

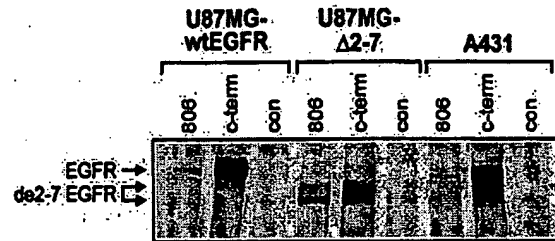


Fig. 2. Immunoprecipitation of EGFR from cell lines. The EGFR was immunoprecipitated from ^{35}S -labeled U87 MG.wtEGFR, U87 MG.Δ2-7, and A431 cells with mAb 806 (806), sc-03 antibody (c-term), or a IgG2b isotype control (con) as described in "Materials and Methods." Arrows, position of the $\Delta 2-7$ and wt EGFR. Identical banding patterns were obtained in three independent experiments.

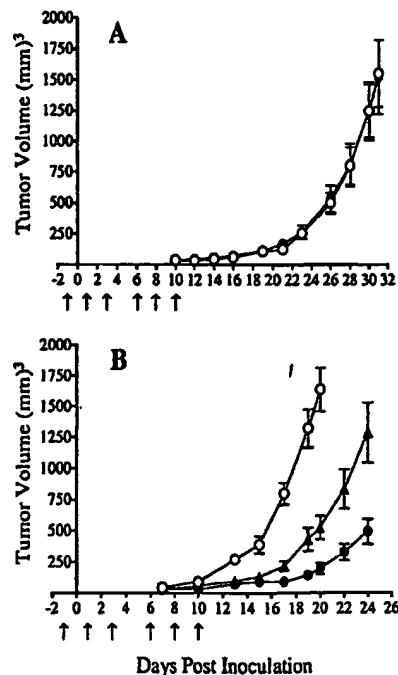


Fig. 3. Antitumor effect of mAb 806 on U87 MG (A) and U87 MG.Δ2-7 (B) xenograft growth rates in a preventative model. U87 MG or U87 MG.Δ2-7 cells (3×10^6) were injected s.c. into both flanks of 4–6-week-old BALB/c nude mice ($n = 5$) at day 0. Mice were injected i.p. with either 1 mg of mAb 806 (●), 0.1 mg of mAb 806 (▲), or vehicle (○) starting 1 day prior to tumor cell inoculation. Injections of mAb 806 were given three times/week for 2 weeks (arrows). Data are expressed as mean tumor volume; bars, SE.

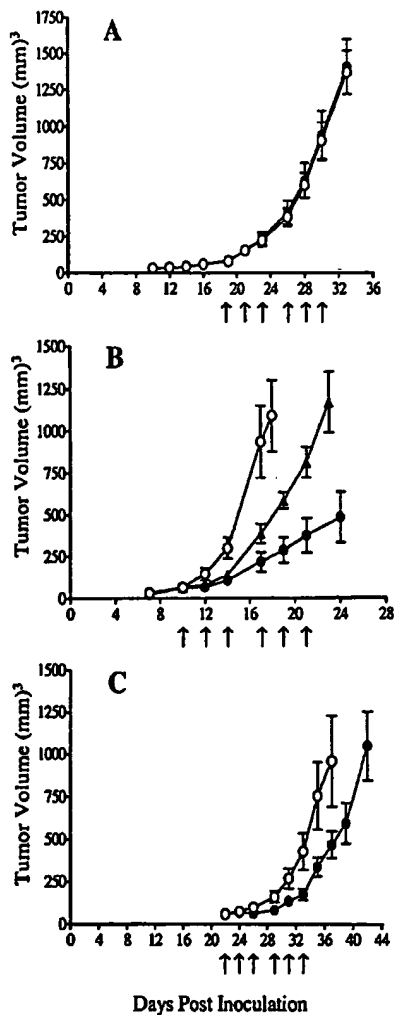


Fig. 4. Antitumor effect of mAb 806 on U87 MG (A), U87 MG.Δ2-7 (B), and U87 MG.wtEGFR (C) xenografts in an established model. U87 MG, U87 MG.Δ2-7, or U87 MG.wtEGFR cells (3×10^6) were injected s.c. into both flanks of 4–6-week-old BALB/c nude mice ($n = 5$). Mice were injected i.p. with either 1-mg doses of mAb 806 (●), 0.1-mg doses of mAb 806 (○), or vehicle (○) starting when tumors had reached a mean tumor volume of 65–84 mm³. Injections were given three times/week for 2 weeks (arrows). Data are expressed as mean tumor volume; bars, SE.

ability to inhibit the growth of established tumor xenografts was examined. Antibody treatment was as described in the preventative model, except that it commenced when tumors had reached a mean tumor volume of 65 mm³ (10 days after implantation) for the U87 MG.Δ2-7 xenografts and 84 mm³ (19 days after implantation) for the parental U87 MG xenografts. Once again, mAb 806 had no effect on the growth of parental U87 MG xenografts, even at a dose of 1 mg/injection (Fig. 4A). In contrast, mAb 806 significantly inhibited the growth of U87 MG.Δ2-7 xenografts in a dose-dependent manner (Fig. 4B). At day 17, 1 day before control animals were sacrificed, the mean tumor volume was 900 ± 200 mm³ for the control group, 400 ± 60 mm³ for the 0.1 mg/injection group ($P < 0.01$), and 220 ± 60 mm³ for the 1 mg/injection group ($P < 0.002$). Treatment of U87 MG.Δ2-7 xenografts with an IgG2b isotype control had no effect on tumor growth (data not shown).

To examine whether the growth inhibition observed with mAb 806 was restricted to cells expressing de2-7 EGFR, its efficacy against the U87 MG.wtEGFR xenografts was also examined in an established

model. These cells serve as a model for tumors containing amplification of the *EGFR* gene without de2-7 EGFR expression. mAb 806 treatment commenced when tumors had reached a mean tumor volume of 73 mm³ (22 days after implantation). mAb 806 significantly inhibited the growth of established U87 MG.wtEGFR xenografts when compared with control tumors treated with vehicle (Fig. 4C). On the day control animals were sacrificed, the mean tumor volume was 1000 ± 300 mm³ for the control group and 500 ± 80 mm³ for the group treated with 1 mg/injection ($P < 0.04$).

Histological and Immunohistochemical Analysis of Established Tumors. To evaluate potential histological differences between mAb 806-treated and control U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts, formalin-fixed, paraffin-embedded sections were stained with H&E (Fig. 5). Areas of necrosis were seen in sections from mAb 806-treated U87 MG.Δ2-7 (mAb 806-treated xenografts were collected 24 days after tumor inoculation and vehicle treated xenografts at 18 days), and U87 MG.wtEGFR xenografts (mAb 806 xenografts were collected 42 days after tumor inoculation and vehicle treated xenografts at 37 days; Fig. 5). This result was consistently observed in a number of tumor xenografts ($n = 4$ for each cell line). However, sections from U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts treated with vehicle ($n = 5$) did not display the same areas of necrosis seen after mAb 806 treatment (Fig. 5). Vehicle and mAb 806-treated xenografts removed at identical times also showed these differences in tumor necrosis (data not shown). Thus, the increase in necrosis observed was not caused by the longer growth periods used for the mAb 806-treated xenografts. Furthermore, sections from mAb 806-treated U87 MG xenografts were also stained with H&E and did not reveal any areas of necrosis (data not shown), further supporting the hypothesis that mAb 806 binding induces decreased cell viability, resulting in increased necrosis within tumor xenografts.

An immunohistochemical analysis of U87 MG, U87 MG.Δ2-7, and U87 MG.wtEGFR xenograft sections was performed to determine the levels of de2-7 and wt EGFR expression after mAb 806 treatment (Fig. 6). As expected, the 528 antibody stained all xenograft sections with no obvious decrease in intensity between treated and control tumors (Fig. 6). Staining of U87 MG sections was undetectable with the mAb 806; however, positive staining of U87 MG.Δ2-7 and U87 MG.wtEGFR xenograft sections was observed (Fig. 6). There was no

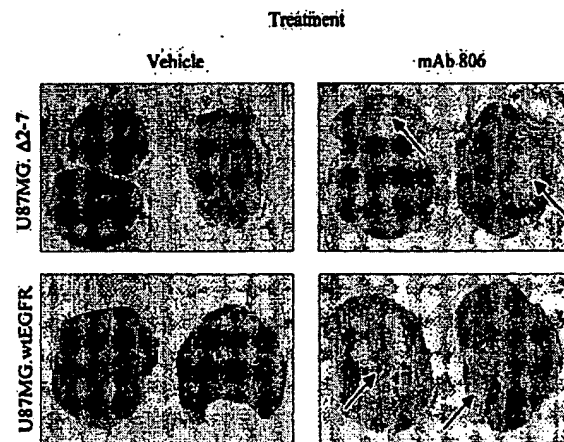
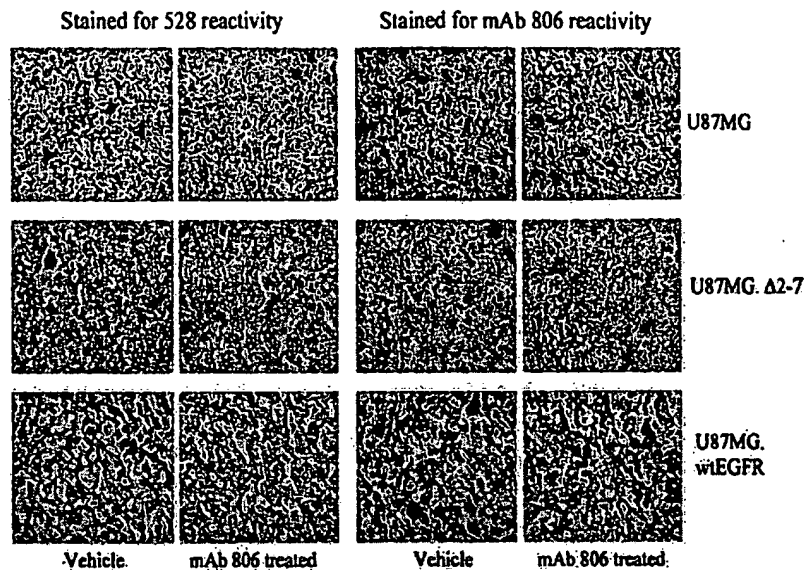


Fig. 5. Representative H&E-stained paraffin sections of U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts. U87 MG.Δ2-7 (collected 24 days after tumor inoculation) and U87 MG.wtEGFR (collected 42 days after tumor inoculation) xenografts were excised from mice treated as described in Fig. 4 and stained with H&E. Vehicle-treated U87 MG.Δ2-7 (collected 18 days after tumor inoculation) and U87 MG.wtEGFR (collected 37 days after tumor inoculation) xenografts showed very few areas of necrosis (left panel), whereas extensive necrosis (arrows) was observed in both U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts treated with mAb 806 (right panel).

Fig. 6. Immunohistochemical analysis of EGFR expression in frozen sections derived from U87 MG, U87 MG.Δ2-7, and U87 MG.wtEGFR xenografts. Sections were collected at the time points described in Fig. 5. Xenograft sections were immunostained with the 528 antibody (left panel) and mAb 806 (right panel). No decreased immunoreactivity to either wt EGFR, amplified EGFR, or de2-7 EGFR was observed in xenografts treated with mAb 806. Consistent with the *in vitro* data, parental U87 MG xenografts were positive for 528 antibody but were negative for mAb 806 staining.



difference in mAb 806 staining intensity between control and treated U87 MG.Δ2-7 and U87 MG.wtEGFR xenografts, suggesting that antibody treatment does not lead to the selection of clonal variants lacking mAb 806 reactivity.

Treatment of A431 Xenografts with mAb 806. To demonstrate that the antitumor effects of mAb 806 were not restricted to U87 MG cells, the antibody was administered to mice containing A431 xenografts. These cells contain an amplified *EGFR* gene and express approximately 2×10^6 receptors/cells. We have previously shown that mAb 806 binds ~10% of these EGFRs and targets A431 xenografts.³ mAb 806 significantly inhibited the growth of A431 xenografts when examined in the preventative xenograft model described previously (Fig. 7A). At day 13, when control animals were sacrificed, the mean tumor volume was $1400 \pm 150 \text{ mm}^3$ in the vehicle-treated group and $260 \pm 60 \text{ mm}^3$ for the 1 mg/injection treatment group ($P < 0.0001$). In a separate experiment, a dose of 0.1 mg of mAb also inhibited significantly ($P < 0.05$) the growth of A431 xenografts in a preventative model (data not shown).

Given the efficacy of mAb 806 in the preventative A431 xenograft model, its ability to inhibit the growth of established tumor xenografts was examined. Antibody treatment was as described in the preventative model, except it was not started until tumors had reached a mean tumor volume of $200 \pm 20 \text{ mm}^3$. mAb 806 significantly inhibited the growth of established A431 xenografts (Fig. 7B). At day 13, the day control animals were sacrificed, the mean tumor volume was $1100 \pm 100 \text{ mm}^3$ for the control group and $450 \pm 70 \text{ mm}^3$ for the 1 mg/injection group ($P < 0.0001$).

Discussion

Many epithelial cancers display increased levels of EGFR expression on the cell surface, and numerous experiments with EGFR mAbs have shown that they inhibit tumor cell growth both *in vitro* and *in vivo* (7, 9, 10, 27). Furthermore, clinical studies using EGFR mAbs in patients with head and neck cancer (28), squamous cell lung cancer (12), gliomas (29), and malignant astrocytomas (30) have all been conducted. However, the clinical use of these antibodies is complicated by liver uptake, as reported in a Phase I clinical trial (12).

An alternative and more specific target for antibody therapy is the tumor-specific de2-7 EGFR, which unlike the wt EGFR, is absent in

normal tissue (4, 14). A number of mAbs have been raised to the unique junctional peptide found in the de2-7 EGFR; these mAbs do not recognize the wt receptor (20-22) and specifically target de2-7 EGFR-positive xenografts grown in nude mice (31, 32). The use of these antibodies should not be complicated by uptake in normal tissues, such as liver and skin. However, with the exception of

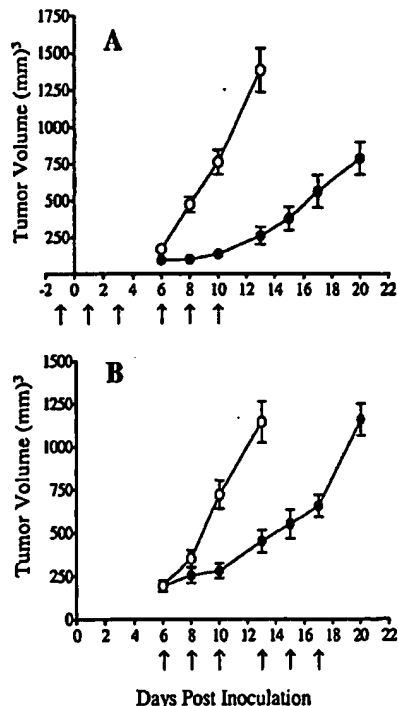


Fig. 7. Antitumor effect of mAb 806 on A431 xenografts in preventative (A) and established (B) models. A431 cells (3×10^6) were injected s.c. into both flanks of 4-6-week-old BALB/c nude mice ($n = 5$). Mice were injected i.p. with either 1-mg doses of mAb 806 (●) or vehicle (○), starting 1 day prior to tumor cell inoculation in the preventative model, or when tumors had reached a mean tumor volume of 200 mm^3 . Injections were given three times/week for 2 weeks (arrows). Data are expressed as mean tumor volume; bars, SE.

gliomas, only a small percentage of most carcinomas express the de2-7 EGFR, apparently restricting the use of such antibodies. We have shown previously² that mAb 806 targets both de2-7 EGFR-transfected U87 MG xenografts and A431 xenografts that overexpress the wt EGFR. mAb 806 did not target parental U87 MG cells, which express $\sim 10^5$ EGFR³ (16). As assessed by FACS, immunohistochemistry, and immunoprecipitation, we now demonstrate that mAb 806 is also able to specifically bind U87 MG.wtEGFR cells, which express $>10^6$ EGFRs/cell. Thus, the previous observed binding of mAb 806 to A431 cells is not the result of some unusual property of these cells but rather appears to be a more general phenomenon related to overexpression of the wt EGFR.

Our data cannot completely exclude the possibility that mAb 806 binds to the wt EGFR with low affinity; however, for several reasons we favor the hypothesis that mAb 806 recognizes a subset of receptors in cells overexpressing the receptor:

(a) We were unable to detect mAb 806 binding to the parental U87 MG cell line, which expresses 1×10^5 wt EGFRs/cell (16), either by FACS, immunoprecipitation, immunohistochemistry, or with iodinated antibody. Indeed, iodinated mAb 806 did not bind to U87 MG cell pellets containing 1×10^7 cells, which based on the Scatchard data using 1×10^6 A431 cells, are conditions that should detect low level antibody binding (i.e., the total number of receptors being similar in both cases).

(b) Scatchard analysis clearly showed that mAb 806 only bound to 10% of the total EGFR on the surface of A431 cells. If mAb 806 simply binds to the wt EGFR with low affinity, then it should have bound to a considerably higher percentage of the receptor.

(c) Comparative immunoprecipitation of the A431 and U87 MG.wtEGFR cell lines with mAb 806 and the sc-03 antibody also supported the hypothesis that only a subset of receptors are recognized by mAb 806. Taken together, these results support the notion that mAb 806 recognizes a EGFR subset on the surface of cells overexpressing the EGFR. We are currently analyzing the EGFR immunoprecipitated by mAb 806 to see if it displays altered biochemical properties related to glycosylation or kinase activity.

The xenograft studies with mAb 806 described here demonstrate dose-dependent inhibition of U87 MG. Δ 2-7 xenograft growth. In contrast, no inhibition of parental U87 MG xenografts was observed, despite the fact that they continue to express the wt EGFR *in vivo*. mAb 806 not only significantly reduced xenograft volume, it also induced significant necrosis within the tumor. As noted above, other de2-7 EGFR-specific mAbs have been generated (20-22), but this is the first report showing the successful therapeutic use of such an antibody *in vivo* against a human de2-7 EGFR-expressing glioma xenograft. A recent report demonstrated that the de2-7 EGFR-specific Y10 mAb had *in vivo* antitumor activity against murine B16 melanoma cells transfected with a murine homologue of the human de2-7 EGFR (33). Y10 mediated *in vitro* cell lysis ($>90\%$) of B16 melanoma cells expressing the de2-7 EGFR in the absence of complement or effector cells. In contrast to their *in vitro* observations, the *in vivo* Y10 antibody efficacy was completely mediated through Fc function when using B16 melanoma cells grown as xenografts in an immunocompetent model. Thus, the direct effects observed *in vitro* do not seem to be replicated when cells are grown as tumor xenografts.

Overexpression of the EGFR has been reported in a number of different tumors and is observed in most gliomas (4, 14). It has been proposed that the subsequent EGFR overexpression mediated by receptor gene amplification may confer a growth advantage by increasing intracellular signaling and cell growth (34). The U87 MG cell line was transfected with the wt EGFR to produce a glioma cell that mimics the process of EGFR gene amplification. Treatment of established U87 MG.wtEGFR xenografts with mAb 806 resulted in sig-

nificant growth inhibition. Thus, mAb 806 also mediates *in vivo* antitumor activity against cells overexpressing the EGFR. Interestingly, mAb 806 inhibition of U87 MG.wtEGFR xenografts was less pronounced than that observed with U87 MG. Δ 2-7 tumors. This probably reflects the fact that mAb 806 has a lower affinity for the overexpressed wt EGFR and only binds a small proportion of receptors expressed on the cell surface.³ However, it should be noted that despite the small effect on U87 MG.wtEGFR xenograft volumes, mAb 806 treatment produced large areas of necrosis within these xenografts. To exclude the possibility that mAb 806 only mediates inhibition of the U87 MG-derived cell lines, we tested its efficacy against A431 xenografts. This squamous cell carcinoma-derived cell line contains significant EGFR gene amplification, which is retained both *in vitro* and *in vivo*. Treatment of A431 xenografts with mAb 806 produced significant growth inhibition in both a preventative and established model, indicating the antitumor effects of mAb 806 are not restricted to transfected U87 MG cell lines.

Complete prevention of A431 xenograft growth by antibody treatment has been reported previously. The wt EGFR mAbs 528, 225, and 425 all prevented the formation of A431 xenografts when administered either on the day or 1 day after tumor inoculation (9, 10). The reason for this difference in efficacy between these wt EGFR antibodies and mAb 806 is not known but may be related to the mechanism of cell growth inhibition. The wt EGFR antibodies function by blocking ligand binding to the EGFR, but this is probably not the case with mAb 806 because it only binds a small EGFR subset on the surface of A431 cells. The significant efficacy of mAb 806 against U87 MG cells expressing the ligand-independent de2-7 EGFR further supports the notion that this antibody mediates its antitumor activity by a mechanism not involving ligand blockade. Therefore, we are currently investigating the nonimmunological and immunological mechanisms that contribute to the antitumor effects of mAb 806. Nonimmunological mechanisms may include subtle changes in receptor levels, blockade of signaling, or induction of inappropriate signaling.

Previously, agents such as doxorubicin and cisplatin in conjunction with wt EGFR antibodies have produced enhanced antitumor activity (35, 36). The combination of doxorubicin and mAb 528 resulted in total eradication of established A431 xenografts, whereas treatment with either agent alone caused only temporary *in vivo* growth inhibition (36). Likewise, the combination of cisplatin and either mAb 528 or 225 also led to the eradication of well-established A431 xenografts, which was not observed when treatment with either agent was used (35). Thus, future studies involving the combination of chemotherapeutic agents with mAb 806 are planned using xenograft models.

Maybe the most important advantage of mAb 806 compared with current EGFR antibodies is that it should be possible to directly conjugate cytotoxic agents to mAb 806. This approach is not feasible with current EGFR-specific antibodies because they target the liver and cytotoxic conjugation would almost certainly induce severe toxicity. Given that mAb 806 failed to bind U87 MG cells expressing 1×10^5 EGFRs and our initial immunohistochemical analysis showing that mAb 806 does not bind normal liver, we believe that it is unlikely this antibody will target normal liver. However, formal demonstration of this requires ongoing analysis and ultimately clinical trials in cancer patients. Conjugation of cytotoxic agents such as drugs (37) or radioisotopes (38) to antibodies has the potential to improve efficacy and reduce the systemic toxicity of these agents. Furthermore, it is likely that the direct antitumor effects of mAb 806 reported here would be further enhanced by the coupling of appropriate cytotoxics.

This study clearly demonstrates that mAb 806 has significant *in vivo* antitumor activity against de2-7 EGFR-positive xenografts and

tumors overexpressing the EGFR. The unique specificity of mAb 806 suggests immunotherapeutic potential in targeting a number of tumor types, particularly head and neck tumors and glioma, without the restrictions associated with normal tissue uptake. Finally, given that systemic administration of mAb 806 inhibits the growth of intracranial glioma xenografts,⁴ we plan to conduct clinical trials with mAb 806 in patients with glioma.

References

- Scott, A. M., and Welt, S. Antibody-based immunological therapies. *Curr. Opin. Immunol.*, 9: 717-722, 1997.
- Fan, Z., and Mendelsohn, J. Therapeutic application of anti-growth factor receptor antibodies. *Curr. Opin. Oncol.*, 10: 67-73, 1998.
- Garcia de Palazzo, I. E., Adams, G. P., Sundaresan, P., Wong, A. J., Testa, J. R., Bigner, D. D., and Weiner, L. M. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res.*, 53: 3217-3220, 1993.
- Wikstrand, C. J., Reist, C. J., Archer, G. E., Zalutsky, M. R., and Bigner, D. D. The class III variant of the epidermal growth factor receptor (EGFRvIII): characterization and utilization as an immunotherapeutic target. *J. Neurovirol.*, 4: 148-158, 1998.
- Korshunov, A., Golanov, A., Sycheva, R., and Pronin, I. Prognostic value of tumour associated antigen immunoreactivity and apoptosis in cerebral glioblastomas: an analysis of 168 cases. *J. Clin. Pathol.*, 52: 574-580, 1999.
- Grundis, J. R., Melhem, M. F., Gooding, W. E., Day, R., Holst, V. A., Wagener, M. M., Drenning, S. D., and Tweardy, D. J. Levels of TGF- α and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J. Natl. Cancer Inst.*, 90: 824-832, 1998.
- Fan, Z., Masui, H., Altas, I., and Mendelsohn, J. Blockade of epidermal growth factor receptor function by bivalent and monovalent fragments of 225 anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res.*, 53: 4322-4328, 1993.
- Teramoto, T., Onda, M., Tokunaga, A., and Asano, G. Inhibitory effect of anti-epidermal growth factor receptor antibody on a human gastric cancer. *Cancer (Phila.)*, 77: 1639-1645, 1996.
- Rodeck, U., Hertly, M., Hertly, D., Molitoff, C., Atkinson, B., Varello, M., Steplewski, Z., and Koprowski, H. Tumor growth modulation by a monoclonal antibody to the epidermal growth factor receptor: immunologically mediated and effector cell-independent effects. *Cancer Res.*, 47: 3692-3696, 1987.
- Masui, H., Kawamoto, T., Sato, J. D., Wolf, B., Sato, G., and Mendelsohn, J. Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res.*, 44: 1002-1007, 1984.
- Faillot, T., Magdelenat, H., Mady, E., Stasielki, P., Fohanno, D., Gropp, P., Poisson, M., and Delattre, J. Y. A Phase I study of an anti-epidermal growth factor receptor monoclonal antibody for the treatment of malignant gliomas. *Neurosurgery*, 39: 478-483, 1996.
- Divgi, C. R., Welt, S., Kris, M., Real, F. X., Yeh, S. D., Gralla, R., Merchant, B., Schweighart, S., Unger, M., Larson, S. M., et al. Phase I and imaging trial of indium 111-labeled anti-epidermal growth factor receptor monoclonal antibody 225 in patients with squamous cell lung carcinoma. *J. Natl. Cancer Inst.*, 83: 97-104, 1991.
- Baselga, J., Pfister, D., Cooper, M. R., Cohen, R., Burtneiss, B., Bos, M., D'Andrea, G., Seidman, A., Norton, L., Gunnert, K., Falcay, J., Anderson, V., Waksal, H., and Mendelsohn, J. Phase I studies of anti-epidermal growth factor receptor chimeric antibody C225 alone and in combination with cisplatin. *J. Clin. Oncol.*, 18: 904, 2000.
- Voldborg, B. R., Damstrup, L., Spang-Thomsen, M., and Poulsen, H. S. Epidermal growth factor receptor (EGFR) and EGFR mutations, function and possible role in clinical trials. *Ann. Oncol.*, 8: 1197-1206, 1997.
- Sugawa, N., Ekstrand, A. J., James, C. D., and Collins, V. P. Identical splicing of aberrant epidermal growth factor receptor transcripts from amplified rearranged genes in human glioblastomas. *Proc. Natl. Acad. Sci. USA*, 87: 8602-8606, 1990.
- Nishikawa, R., Ji, X. D., Harmon, R. C., Lazar, C. S., Gill, G. N., Cavenee, W. K., and Huang, H. J. A mutant epidermal growth factor receptor common in human glioma confers enhanced tumorigenicity. *Proc. Natl. Acad. Sci. USA*, 91: 7727-7731, 1994.
- Tang, C. K., Gong, X. Q., Moscatello, D. K., Wong, A. J., and Lippman, M. E. Epidermal growth factor receptor vIII enhances tumorigenicity in human breast cancer. *Cancer Res.*, 60: 3081-3087, 2000.
- Olapade-Olaopa, E. O., Moscatello, D. K., MacKay, E. H., Horsburgh, T., Sandhu, D. P., Terry, T. R., Wong, A. J., and Habib, F. K. Evidence for the differential expression of a variant EGF receptor protein in human prostate cancer. *Br. J. Cancer*, 82: 186-194, 2000.
- Moscatello, D. K., Holgado-Madruga, M., Godwin, A. K., Ramirez, G., Gurn, G., Zolnick, P. W., Biegel, J. A., Haycs, R. L., and Wong, A. J. Frequent expression of a mutant epidermal growth factor receptor in multiple human tumors. *Cancer Res.*, 55: 5536-5539, 1995.
- Wikstrand, C. J., Hale, L. P., Batra, S. K., Hill, M. L., Humphrey, P. A., Kurpad, S. N., McLendon, R. E., Moscatello, D., Pegram, C. N., Reist, C. J., et al. Monoclonal antibodies against EGFRvIII are tumor specific and react with breast and lung carcinomas and malignant gliomas. *Cancer Res.*, 55: 3140-3148, 1995.
- Hills, D., Rowlinson-Busza, G., and Gullick, W. J. Specific targeting of a mutant activated FGF receptor found in glioblastoma using a monoclonal antibody. *Int. J. Cancer*, 63: 537-543, 1995.
- Okamoto, S., Yoshikawa, K., Ohta, Y., Shibuya, M., Aoki, S., Yoshida, J., and Takahashi, T. Monoclonal antibody against the fusion junction of a deletion-mutant epidermal growth factor receptor. *Br. J. Cancer*, 73: 1366-1372, 1996.
- Nagane, M., Coufal, F., Lin, H., Bogler, O., Cavenee, W. K., and Huang, H. J. A common mutant epidermal growth factor receptor confers enhanced tumorigenicity on human glioblastoma cells by increasing proliferation and reducing apoptosis. *Cancer Res.*, 56: 5079-5086, 1996.
- Sato, J. D., Le, A. D., and Kawamoto, T. Derivation and assay of biological effects of monoclonal antibodies to epidermal growth factor receptors. *Methods Enzymol.*, 146: 63-81, 1987.
- Huang, H. S., Nagane, M., Klingbeil, C. K., Lin, H., Nishikawa, R., Ji, X. D., Huang, C. M., Gill, G. N., Wiley, H. S., and Cavenee, W. K. The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling. *J. Biol. Chem.*, 272: 2927-2935, 1997.
- Clarke, K., Lee, F. T., Brechbiel, M. W., Smyth, F. E., Old, L. J., and Scott, A. M. Therapeutic efficacy of anti-Lewis(y) humanized 3S193 radioimmunotherapy in a breast cancer model: enhanced activity when combined with Taxol chemotherapy. *Clin. Cancer Res.*, 6: 3621-3628, 2000.
- Atlas, I., Mendelsohn, J., Baselga, J., Fair, W. R., Masui, H., and Kumar, R. Growth regulation of human renal carcinoma cells: role of transforming growth factor α . *Cancer Res.*, 52: 3335-3339, 1992.
- Perez-Soler, R., Donato, N. J., Shin, D. M., Rosenblum, M. G., Zhang, H. Z., Tornos, C., Brewer, H., Chan, J. C., Lee, J. S., Hong, W. K., et al. Tumor epidermal growth factor receptor studies in patients with non-small-cell lung cancer or head and neck cancer treated with monoclonal antibody RG 83852. *J. Clin. Oncol.*, 12: 730-739, 1994.
- Wersall, P., Ohlsson, I., Biberfeld, P., Collins, V. P., von Krusenstjerna, S., Larsson, S., Mellstedt, H., and Boethius, J. Intratumoral infusion of the monoclonal antibody, mAb 425, against the epidermal-growth-factor receptor in patients with advanced malignant glioma. *Cancer Immunol. Immunother.*, 44: 157-164, 1997.
- Brady, L. W., Miyamoto, C., Woo, D. V., Rackover, M., Emrich, J., Bender, H., Dadgar, S., Steplewski, Z., Koprowski, H., Black, P., et al. Malignant astrocytomas treated with iodine-125 labeled monoclonal antibody 425 against epidermal growth factor receptor: a Phase II trial. *Int. J. Radiat. Oncol. Biol. Phys.*, 22: 225-230, 1992.
- Reist, C. J., Archer, G. E., Kurpad, S. N., Wikstrand, C. J., Vaidyanathan, G., Willingham, M. C., Moscatello, D. K., Wong, A. J., Bigner, D. D., and Zalutsky, M. R. Tumor-specific anti-epidermal growth factor receptor variant III monoclonal antibodies: use of the tyramine-cellobiose radioiodination method enhances cellular retention and uptake in tumor xenografts. *Cancer Res.*, 55: 4375-4382, 1995.
- Reist, C. J., Archer, G. E., Wikstrand, C. J., Bigner, D. D., and Zalutsky, M. R. Improved targeting of an anti-epidermal growth factor receptor variant III monoclonal antibody in tumor xenografts after labeling using *N*-succinimidyl 5-iodo-3-pyridinecarboxylate. *Cancer Res.*, 57: 1510-1515, 1997.
- Sampson, J. H., Crotty, L. E., Lee, S., Archer, G. E., Ashley, D. M., Wikstrand, C. J., Hale, L. P., Small, C., Dranoff, G., Friedman, A. H., Friedman, H. S., and Bigner, D. D. Unarmed, tumor-specific monoclonal antibody effectively treats brain tumors. *Proc. Natl. Acad. Sci. USA*, 97: 7503-7508, 2000.
- Films, J., Trent, J. M., Pollak, M. N., and Buick, R. N. Epidermal growth factor receptor gene-amplified MDA-468 breast cancer cell line and its nonamplified variants. *Mol. Cell. Biol.*, 7: 251-257, 1987.
- Fan, Z., Baselga, J., Masui, H., and Mendelsohn, J. Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus *cis*-diamminedichloroplatinum on well established A431 cell xenografts. *Cancer Res.*, 53: 4637-4642, 1993.
- Baselga, J., Norton, L., Masui, H., Pandiella, A., Coplan, K., Miller, W. H., Jr., and Mendelsohn, J. Antitumor effects of doxorubicin in combination with anti-epidermal growth factor receptor monoclonal antibodies. *J. Natl. Cancer Inst.*, 85: 1327-1333, 1993.
- Trail, P. A., and Bianchi, A. B. Monoclonal antibody drug conjugates in the treatment of cancer. *Curr. Opin. Immunol.*, 11: 584-588, 1999.
- DeNardo, S. J., Kroger, L. A., and DeNardo, G. L. A new era for radiolabeled antibodies in cancer? *Curr. Opin. Immunol.*, 11: 563-569, 1999.

⁴ K. Mishima, T. G. Johns, A. M. Scott, E. Stockert, X-D. Ji, P. Suvama, J. R. Voland, L. J. Old, H.-J. S. Huang, and W. K. Cavenee. Growth suppression of intracranial xenografted glioblastomas overexpressing mutant epidermal growth factor receptors by systemic administration of mAb 806, a novel monoclonal antibody directed to the receptor; submitted for publication.



IMMUNOHISTOCHEMICAL COMPARATIVE ANALYSIS OF TRANSFORMING GROWTH FACTOR α , EPIDERMAL GROWTH FACTOR, AND EPIDERMAL GROWTH FACTOR RECEPTOR IN NORMAL, HYPERPLASTIC AND NEOPLASTIC HUMAN PROSTATES

María P. De Miguel,¹ Mar Royuela,¹ Fermín R. Bethencourt,² Antonio Ruiz,³ Benito Fraile,¹ Ricardo Paniagua¹

Immunoreaction to TGF- α was limited to the basal epithelial cells of focal areas in the normal prostates. In benign prostatic hyperplasia (BPH) the immunostained areas were more widespread and immunolabelling was observed in both basal and columnar (secretory) cells of the epithelium. Some cells in the connective tissue stroma were also stained. In prostatic adenocarcinoma, epithelial immunostaining was even more extensive and intense than in BPH, and some stromal cells were also stained. Epidermal growth factor (EGF) immunostaining was only present in some basal cells in normal prostates. In BPH, this immunoreaction was strong in the basal cells and even stronger in the secretory cells. In prostatic cancer, the intensity of epithelial cell immunoreactivity was intermediate between that of normal prostates and that of BPH specimens. EGF-receptor immunostaining was focal and located in the basal cells in normal prostates. In BPH, labelling was also localized in basal cells but extended to wider areas. Some stromal cells appeared weakly labelled. In the prostatic carcinoma, both basal and columnar cells appeared stained and the number of immunolabelled stromal cells was higher than in BPH. The results presented suggest that, in normal conditions, EGF and TGF- α act as autocrine growth factors for the basal cells of the prostatic epithelium. In BPH this action is maintained and, in addition, the columnar cells start to secrete both factors which are bound by the basal cell receptors, giving rise to a paracrine regulation which probably overstimulates basal cell proliferation. In prostatic carcinoma, besides these regulatory mechanisms, the acquisition of EGF-receptors by the secretory cells develops an autocrine regulation which might induce their proliferation.

© 1999 Academic Press

Epidermal growth factor (EGF) is a 6-kDa polypeptide¹ which stimulates proliferation and keratinization of epithelial tissues in vivo and in vitro conditions.² It is produced by a variety of normal cells in different tissues and has been isolated in most

organic fluids including human urine,³ prostatic secretion and seminal fluid.⁴ Transforming growth factor α (TGF- α) is a 5-kDa polypeptide which shares 35% of its amino acid sequence with EGF.^{5,6} Unlike EGF, TGF- α is produced by malignant cells, including those transfected by viruses.⁷ The EGF-receptor (EGFR) is an intrinsic membrane glycoprotein of 170 kDa.⁸ The use of the same receptor by TGF- α with a similar affinity⁹ implies the intracellular transduction of the same signal.¹⁰

Prostatic epithelial cells synthesize EGF¹¹ and TGF- α .¹² Immunohistochemical localization of EGF in benign prostatic hyperplasia (BPH) and prostate cancer was reported by Fowler *et al.*¹³ EGFRs have been identified in the cytoplasm of normal human prostate cells¹⁴ and in the membrane fraction of prostatic tissue from men with BPH¹⁵ or prostatic

From the ¹Department of Cell Biology and Genetics, University of Alcalá; ²Department of Urology, Hospital Principe de Asturias; ³Department of Pathology, Hospital Principe de Asturias, E-28871 Alcalá de Henares, (Madrid), Spain

Correspondence to: Ricardo Paniagua, Department of Cell Biology and Genetics, University of Alcalá, E-28871 Alcalá de Henares, Madrid, Spain; E-mail: bcrpg@gene.alcala.es

Received 29 January 1998; received in revised form 11 August 1998; accepted for publication 30 September 1998

© 1999 Academic Press

1043-4666/99/090722+06 \$30.00/0

KEY WORDS: benign prostatic hyperplasia/epidermal growth factor (EGF)/human prostate/prostatic cancer/transforming growth factor α (TGF- α)

TABLE 1. Comparison of immunostaining intensities of the prostatic epithelium (measured as optical density) in normal prostate, benign prostatic hyperplasia, and prostatic adenocarcinoma

Immunostaining	Normal men	Benign prostatic hyperplasia	Prostatic adenocarcinoma
TGF- α	5.40 \pm 1.1 ^a	8.04 \pm 2.2 ^b	8.30 \pm 2.4 ^b
EGF	0.72 \pm 0.1 ^a	4.49 \pm 0.9 ^b	2.96 \pm 1.2 ^c
EGFR	5.70 \pm 1.5 ^a	6.35 \pm 2.3 ^a	8.04 \pm 2.0 ^b

For each immunostaining, values with different superscript letters differ significantly between them ($P \leq 0.05$).

carcinoma.¹⁶ Comparative immunohistochemical studies on these growth factors in normal prostate, BPH and prostatic carcinoma are partial for reasons that include: the lack of normal prostatic tissue for comparison purposes, the impossibility of using an animal model, since laboratory animals do not develop prostatic carcinoma, and our relatively poor knowledge of the growth factors and their receptors that control the normal development of the prostate.

RESULTS

The results from tests used to check the specificity of the three antibodies used were positive. No immunoreaction was observed in the negative controls incubated with pre-immune serum or anti-chloramphenicol. Staining of skin sections was positive. The results of ELISA showed a linear correlation between the increasing concentrations of the homogenized tissues and their respective optical densities. Comparison of prostates obtained during surgery with those from autopsies showed neither histological nor immunohistochemical changes. In the normal prostates, no differences in immunostaining affinity were observed between prostatic regions (central, intermediate and peripheral).

The results of the semiquantitative comparative analysis are summarized in Table 1.

In the normal prostates, immunoreaction to TGF- α was limited to focal areas that appeared intermingled with the unstained areas. In the stained areas, labelling was only observed in the basal epithelial cells (Fig. 1). In BPH, the immunostained areas were more widespread and immunolabelling was observed in both basal and columnar (secretory) cells of the epithelium. Some cells in the connective tissue stroma were also stained (Fig. 2). In prostatic adenocarcinoma immunostaining was even more extensive. Although distinction between basal cells and columnar cells were less evident than in the other groups, all the epithelial

cells and some stromal cells were intensely immunostained (Fig. 3).

In normal prostates, EGF immunostaining was absent or only present in some basal cells (Fig. 4). In contrast, in BPH this immunoreaction was strong in the basal cells and even stronger in the secretory cells of the prostatic epithelium (Fig. 5). Prostatic secretions also appeared intensely labelled. In prostatic cancer sections, the EGF immunoreactivity of the epithelial cells was more intense than in normal prostates and weaker than in BPH (Fig. 6). No positive immunoreaction to EGF was observed in the stroma in any of the three prostate types studied.

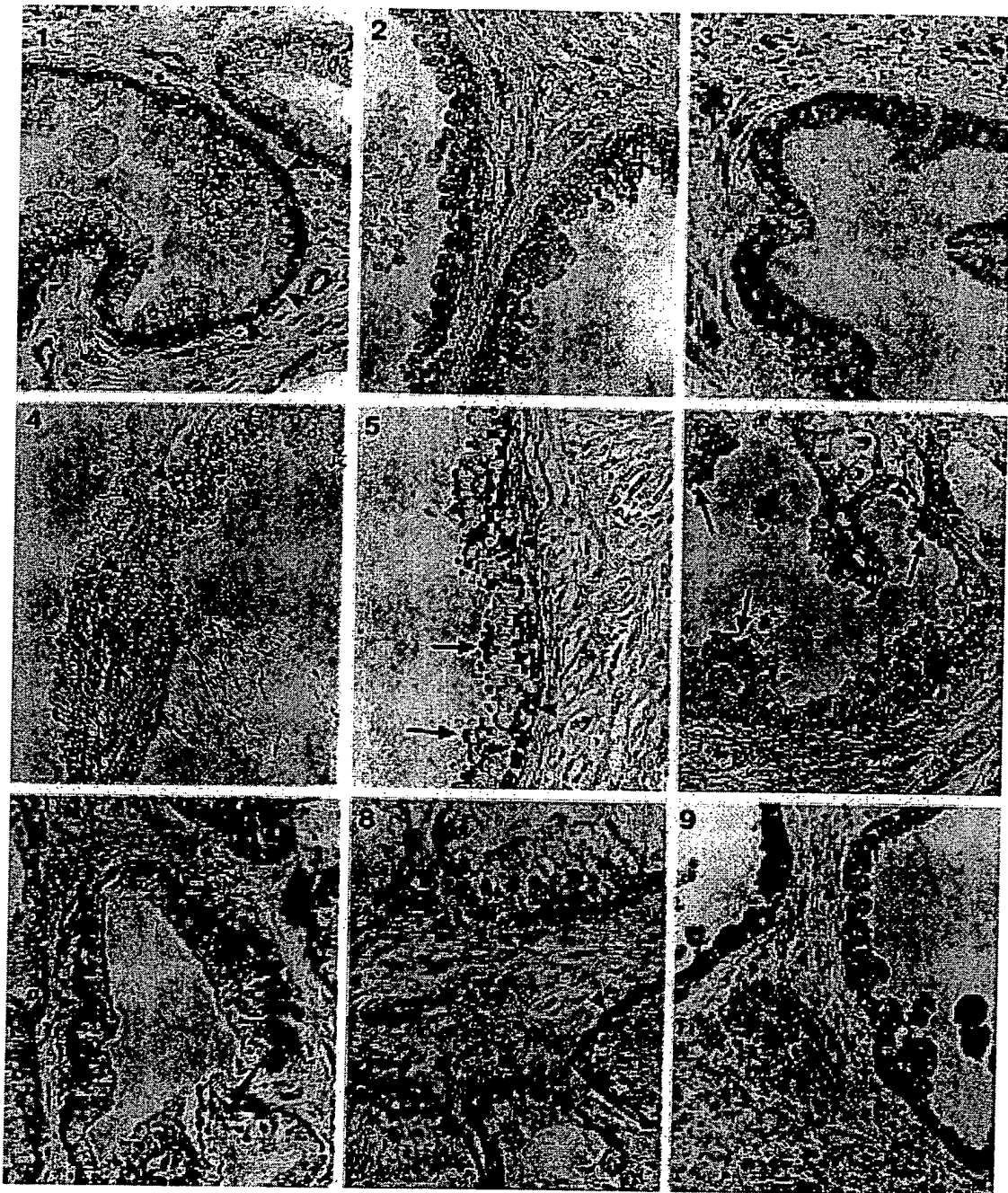
In normal prostates, immunostaining to EGFR only appeared in some zones, that were intermingled with the unstained areas and was exclusively localized in the basal epithelial cells (Fig. 7). In BPH, labelling was also in basal cells but extended to wider areas. In addition, some stromal cells appeared weakly labelled (Fig. 8). In the prostatic carcinoma specimens, the immunolabelled areas were even wider and all the epithelial cells in these areas appeared intensely stained. In the connective tissue stroma, the number of immunolabelled cells was higher than in BPH and their staining was more intense (Fig. 9).

DISCUSSION

Harper *et al.*¹⁷ found that immunohistochemical reaction to TGF- α was very low in BPH and increased in prostate cancer. In the normal prostates evaluated in this study, TGF- α protein was expressed exclusively in the basal epithelial cells. In BPH the columnar secretory cells were also labelled. In prostatic carcinoma immunostaining was more intense than in BPH. This agrees with the opinion that autocrine production of TGF- α is characteristic of prostatic tumour cells.¹⁸

Previous results on EGF in BPH and prostatic cancer are controversial and in any of them comparisons with normal prostatic tissue have been performed. Using immunohistochemistry, Fowler *et al.*¹³ identified EGF staining in the prostate only in 6% of BPH patients compared with 68% of prostate cancer patients. In contrast, radioimmunoassay quantitations by Habib¹⁹ and Shaikh *et al.*²⁰ found significantly higher levels of EGF in patients with BPH than in prostatic cancer patients. These results agree with those reported here.

The results of previous studies on EGFR in BPH and prostatic carcinoma are also discrepant. Several authors²¹⁻²³ have reported that the number of EGFR positive cells is higher in BPH than in prostate carcinoma. In contrast, in tumour cell line cultures, EGFR expression increases with malignant potential^{24,25} and



Figures 1-9. Paraffin embedded sections of the prostate immunostained to TGF- α (Figs 1-3), to EGF (Figs 4-6) or to EGFR (Figs 7-9).
 Figure 1: In the normal prostate the basal epithelial cells (arrowheads) are intensely immunostained. $\times 425$.
 Figure 2: Both basal cells and columnar secretory cells are stained in BPH. $\times 330$.
 Figure 3: In prostatic adenocarcinoma epithelial immunostaining is more intense and some stroma cells (star) are also stained. $\times 240$.
 Figure 4: In the normal prostate epithelial immunostaining is weak and limited to some basal (arrowheads) cells. $\times 320$.
 Figure 5: Basal cells (arrowheads) and columnar cells (arrows) are stained in BPH. $\times 240$.
 Figure 6: In prostatic adenocarcinoma epithelial cells are also immunostained (arrows) but labelling is less intense than in BPH. $\times 20$.
 Figure 7: In the normal prostate the basal epithelial cells are intensely immunostained (arrowheads). $\times 310$.
 Figure 8: In addition to basal cells (arrowheads), some stromal cells (star) are immunostained in BPH. $\times 500$.
 Figure 9: The epithelium and some stromal cells (star) are intensely immunostained in prostatic adenocarcinoma. $\times 300$.
 Reproduced at 85%.

EGFR mRNA levels were found to be slightly higher in prostatic cancer patients than in BPH.^{23,26} In other studies, the levels of EGF binding were similar in both

groups.²⁷ In the present study, the immunostaining pattern of EGF receptor differs from that of their ligands, and the intensity of EGFR immunostaining

was higher in prostatic carcinoma than in BPH and normal prostates.

There have been some studies on the intra-epithelial localization of EGFR. In an immunohistochemical study of BPH, prostatic intra-epithelial neoplasia (PIN) and prostatic carcinoma, Ibrahim

*et al.*²¹ and Maddy *et al.*²⁸ observed that EGFR immunoreactivity only appeared in the basal cells of the prostatic epithelium in both BPH and prostate cancer. In the present study, in the normal prostate and BPH, immunostaining to this receptor was only seen in basal cells whereas, in the prostatic carcinoma, the whole epithelium and some stromatic cells were strongly immunostained. Cohen *et al.*²⁹ found that, in normal prostates and BPH specimens, EGFR immunostaining was localized in the epithelial cells and TGF- α in the stromal cells, whereas in about half of the adenocarcinomas examined there was co-expression of the receptor and the ligand. Scher *et al.*³⁰ found this co-expression in epithelial tumour cells but only in specimens obtained from hormone-refractory metastases and not in primary prostatic tumours. These findings suggest that in primary tumours a paracrine pattern of growth factor stimulation predominates, whereas in androgen-independent disease a shift towards an autocrine stimulatory loop occurs.

The results reported here suggest the following hypothesis. In normal conditions, EGF and TGF- α act as autocrine growth factors for the basal cells of the prostatic epithelium (Fig. 10A). In BPH this action is maintained and, in addition, the columnar cells start to secrete both factors which are bound by the basal cells, giving rise to a paracrine regulation which probably overstimulates basal cell proliferation (Fig. 10B). In prostatic carcinoma, besides these regulatory mechanisms, the acquisition of EGFRs by the secretory cells develops an autocrine regulation which might induce their proliferation (Fig. 10C).

This interpretation is supported by previous data. It has been suggested that expression of TGF- α and its receptor by human prostate cancer cells could confer them a growth advantage, leading to its autonomous growth.⁷ A marked epithelial dysplasia resembling carcinoma-in-situ cells has been observed in the anterior prostate of transgenic mice overexpressing TGF- α ,³¹ and suggests that overproduction of this factor contributes to continuous cell proliferation and transformation.³² To achieve transformation, EGFR

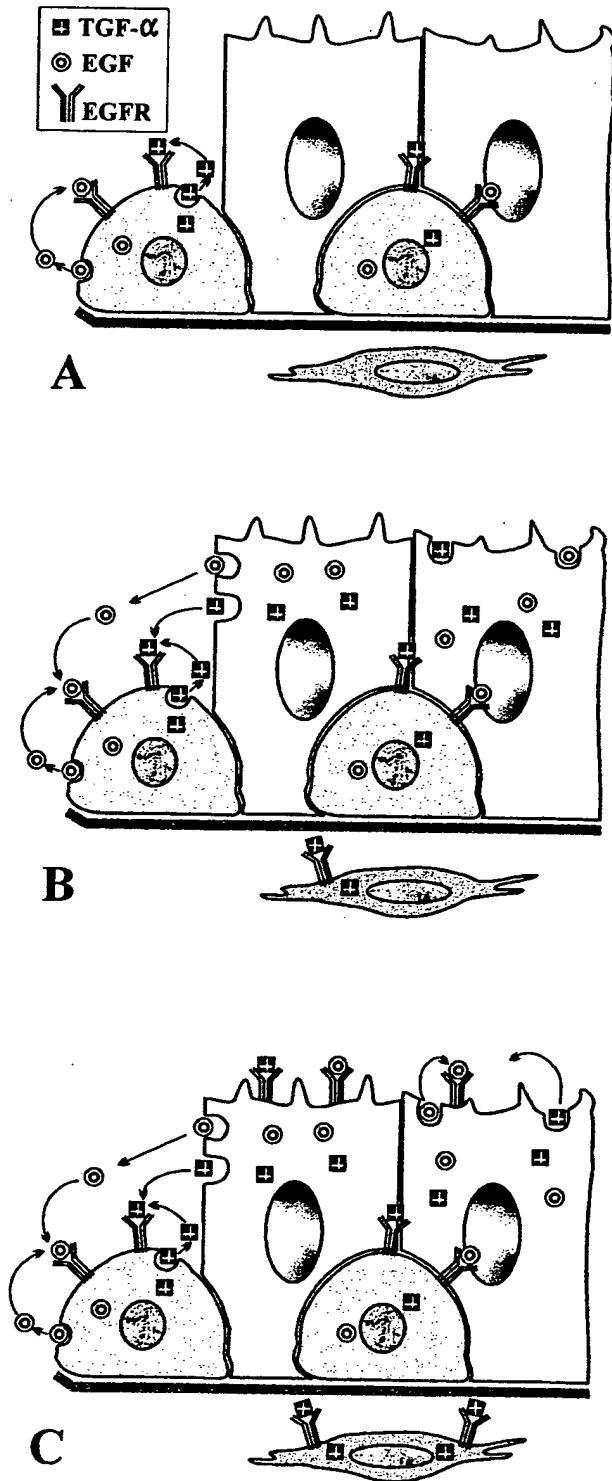


Figure 10. Possible actions of TGF- α , EGF and EGFR on the human prostate.

A: In normal conditions, TGF- α and EGF are secreted by basal epithelial cells which also possess receptors for these factors (EGFR), giving rise to an autocrine stimulation of basal cell proliferation (epithelial cell renewal). B: In benign prostatic hyperplasia, the columnar secretory cells also secrete these factors, giving rise to a paracrine overstimulation of basal cells. The excessive proliferation of these cells might be the cause of hyperplasia. C: In prostatic adenocarcinoma, in addition to the autocrine and paracrine stimulations of basal cells, the columnar cells—which have developed EGFRs—undergo an autocrine stimulation and thus, proliferation of the usually non-proliferative columnar cells also might occur.

must be expressed at high levels in addition to being in the presence of activating ligand.³³ In our study, EGFR is overexpressed in all epithelial cells of the prostatic epithelium in the cancer samples. A mechanism for hyperproliferation is an unscheduled autocrine ligand synthesis by pre-malignant cells.³⁴ In our study, an important ligand might be TGF- α , which appears in the secretory cells of BPH specimens while it is completely absent in normal prostate and increases to higher levels in prostatic carcinoma.

MATERIALS AND METHODS

The prostates from 30 men (aged from 60 to 80 years) were obtained by surgery. Fifteen of these men were clinically and histopathologically diagnosed of BPH, and the other 15 men presented prostatic adenocarcinoma (Gleason grading 3). In addition, 10 prostates from 20-50-year-old men without reproductive, endocrine and related diseases were obtained between 8 and 10 h after death in autopsies. Five of these men showed BPH and the other five men presented histologically normal prostates.

Tissues were fixed for 24 h in a 0.1 M phosphate-buffered 10% formaldehyde solution, dehydrated and embedded in paraffin. Sections (5 μ m in thickness) were processed following the alkaline phosphatase-conjugated streptavidin complex or the avidin-biotin-peroxidase complex methods. In brief, following deparaffinization, sections were hydrated and, in the case of the avidin-biotin-peroxidase complex method, incubated for 20 min in 0.3% H₂O₂ in methanol to reduce endogenous peroxidase activity. The sections were incubated overnight at 4°C with the primary antibodies (all from Santa Cruz Biotechnologies, Santa Cruz, CA, USA) diluted in Tris-buffered saline (TBS) containing 0.5% BSA. The primary antibodies used and the dilutions found to be optimal for this study were: anti-EGF antibody, at 1:25; anti-TGF- α antibody, at 1:25; and anti-EGFR antibody, at 1:250. Afterwards, the sections were washed twice in TBS and then incubated with goat anti-rabbit (EGF and EGFR) or goat anti-mouse (TGF- α) biotinylated immunoglobulins (Biogenex, San Ramon, CA, USA). After 1 h of incubation at 37°C with the second antibody, the sections were incubated with phosphatase-antiphosphatase complex (Biogenex) or avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature, developed with Fast Red (Biogenex-supersensitivity complex Kit) or with diaminobenzidine (Sigma, Barcelona, Spain), and mounted in Crystal/mount aqueous dry mounting medium (Biomed, Foster City, CA, USA) or dehydrated and mounted in DePex (Probus, Badalona, Spain). Afterwards, some sections were counterstained with Mayer's haematoxylin for 2 min. Care was always taken to develop the sections of the different specimens for exactly the same time.

The specificity of the immunohistochemical procedures was checked by using negative and positive control sections. For negative control of the immunoreactions, adjacent sections of each type (normal, BPH and prostatic cancer)

were incubated with pre-immune rabbit serum (for EGF and EGFR) or mouse anti-chloramphenicol (for TGF- α , a gift from P. J. S. van Kooten, Institute of Infectious Diseases and Immunology, University of Utrecht, Utrecht, The Netherlands). As positive controls, sections of skin from both mouse and human were incubated with the same antibodies. For additional control of the specificity of the antibodies, an ELISA was carried out. The protein concentration of homogenates was calculated by the Bradford method.³⁵ The different antigens were coated on 96 well multiplates overnight at 4°C. The plates were washed with TBS containing 0.05% Tween 20 and blocked with 1% bovine serum albumin in TBS for 1 h at room temperature, and incubated with the different antibodies for 3 h also at room temperature. After a further wash, the peroxidase-conjugated anti-rabbit (EGF, EGFR) or anti-mouse (TGF- α) immunoglobulins were added to each well (Chemicon, Temecula, CA, USA). The interactions were visualized with 0.5% 2,2-azino-di-3-ethylbenzothiazolone sulphonic acid (ABTS) (Sigma) in 100 mM citrate buffer, and was measured (optical density at 405 nm) in a spectrophotometer (Multiskan Bichromatic, Labsystems, Finland).

A histological comparative quantification of immunolabelling density in normal, hyperplastic and neoplastic prostates was performed for each of the three antibodies. Of each normal prostate, six histological sections of each region (central, intermediate and peripheral) were selected at random and the staining intensity (optical density) per unit surface area of the epithelium was measured with an automatic image analyser (MIP4 version 4.4, Consulting Image Digital, Barcelona, Spain) in five light microscopic fields using the $\times 40$ objective. For each positively immunostained section one control section (the following in a series of consecutive sections) was also used, and the optical density of this control section was subtracted from that of the stained section. The means \pm SD for the normal prostate group were calculated from the average values obtained for each prostate. The same quantitative study was carried out in the hyperplastic and neoplastic prostates, although the number of sections used was higher (23 in BPH and 29 in prostatic carcinoma) and all these sections were taken from the impaired zone. In the study, the number of sections and microscopic fields in each section necessary for calculation were determined by successive approaches to obtain the minimum number required to reach the lowest SD. The statistical significance between means was assessed by the Fisher and Behrens test.

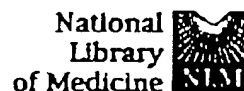
Acknowledgements

This work was supported by grants from the Comunidad de Madrid (AC-095), the Fondo de Investigaciones Sanitarias (98/0820) and the University of Alcalá (003/97).

REFERENCES

1. Cohen S (1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. *J Biol Chem* 237:1555-1562.

2. Gregory H (1985) In vivo aspects of urogastrone-epidermal growth factor. *J Cell Sci Suppl.* 3:11-17.
3. Cohen S, Carpenter G (1975) Human epidermal growth factor: isolation of chemical and biological properties. *Proc Natl Acad Sci USA* 72:1317-1321.
4. Carpenter G, Wahl M (1990) The epidermal growth factor family. In Sporn MB and Roberts AB (eds) *Peptide growth factors and their receptors*, Springer, Heidelberg, pp. 69-171.
5. Marquardt H, Hunkapiller MW, Hood LE, Twardzik DK, De Larco JR, Stephenson JR, Todaro GJ (1983) Transforming growth factors produced by retrovirus-transformed rodent fibroblasts and human melanoma cells: amino acid sequence homology with epidermal growth factor. *Proc Natl Acad Sci USA* 80:4684-4688.
6. Derynck R (1986) Transforming growth factor- α : structure and biological activities. *J Cell Biochem* 32:203-204.
7. Story M (1991) Polypeptide modulators of prostatic growth and development. *Cancer Surveys* 11:123-146.
8. Hunter T (1984) The epidermal growth factor receptor gene and its product. *Nature* 311:414-416.
9. Carpenter G (1987) Receptors for epidermal growth factor and other polypeptide mitogens. *Ann Rev Biochem* 56:881-914.
10. Reynolds FH Jr, Todaro GJ, Fryling C, Stephenson JR (1981) Human transforming growth factors induces tyrosine phosphorylation of EGF receptors. *Nature* 292:259-262.
11. Gregory H, Willshire IR, Kavanagh JP, Blacklock NJ, Clowdury S, Richards RC (1986) Urogastrone-epidermal growth factor concentrations in prostatic fluid of normal individuals and patients with benign prostatic hypertrophy. *Clin Sci* 70:359-363.
12. Taylor TB, Ramsdell JS (1993) Transforming growth factor- α and its receptor are expressed in the epithelium of the rat prostate gland. *Endocrinol* 133:1306-1311.
13. Fowler JE Jr, Lau JLT, Grosh L, Mills SE, Mounzer A (1988) Epidermal growth factor and prostatic carcinoma: an immunohistochemical study. *J Urol* 139:857-861.
14. Damjanov I, Mildner B, Knowles BB (1986) Immunohistochemical localization of the epidermal growth factor receptor in normal human tissues. *Lab Invest* 55:588-592.
15. Maddy SQ, Chisholm GD, Hawkins RA, Habib FK (1987) Localization of epidermal growth factor receptors in the human prostate by biochemical and immunocytochemical methods. *J Endocrinol* 113:147-153.
16. Eaton CL, Davies P, Phillips MEA (1988) Growth factor involvement and oncogene expression in prostatic tumours. *J Steroid Biochem* 30:341-345.
17. Harper ME, Goddard L, Glynn-Jones E, Wilson DW, Price-Thomas M, Peeling WB, Griffiths K (1993) An immunocytochemical analysis of TGF α expression in benign and malignant prostatic tumors. *Prostate* 23:9-23.
18. Wilding G, Valverius E, Knabbe C, Gelmann E (1989) Role of transforming growth factor- α in human prostate cancer cell growth. *Prostate* 15:1-12.
19. Habib FK (1990) Peptide growth factors: a new frontier in prostate cancer. *Prog Clin Biol Res* 357:107-115.
20. Shaikh N, Lai L, McLoughlin J, Clark D, Williams G (1990) Quantitative analysis of epidermal growth factor in human benign prostatic hyperplasia and prostatic carcinoma and its prognostic significance. *Anticancer Res* 10:873-874.
21. Ibrahim FK, Kerns B-JM, MacDonald JA, Ibrahim SN, Kinney RB, Humphrey PA, Robertson CN (1993) Differential immunoreactivity of epidermal growth factor receptor in benign, dysplastic and malignant prostatic tissues. *J Urol* 149:170-173.
22. Maygarden SJ, Strom S, Ware JL (1992) Localization of epidermal growth factor receptor by immunohistochemical methods in human prostatic carcinoma, prostatic intraepithelial neoplasia, and benign hyperplasia. *Arch Pathol Lab Med* 116:269-273.
23. Turkeri LN, Sakr WA, Wykes SM, Grignon DJ, Pontes JE, Macoska JA (1994) Comparative analysis of epidermal growth factor receptor gene expression and protein product in benign, premalignant and malignant prostate tissue. *Prostate* 25:199-205.
24. Davidson NE, Gelmann EP, Lippman ME, Dickson RB (1987) Epidermal growth factor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. *Mol Endocrinol* 1:216-223.
25. MacDonald A, Habib FK (1992) Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. *Br J Cancer* 65:177-182.
26. Morris GL, Dodd JG (1990) Epidermal growth factor receptor mRNA levels in human prostatic tumors and cell lines. *J Urol* 143:1272-1274.
27. Davis P, Eaton CL (1989) Binding of epidermal growth factor by human normal, hypertrophic, and carcinomatous prostate. *Prostate* 14:123-132.
28. Maddy SQ, Chisholm GD, Bussuttil A, Habib FK (1989) Epidermal growth factor receptors in human prostate cancer: correlation with histological differentiation of the tumour. *Br J Cancer* 60:41-44.
29. Cohen S, Simak R, Fair WR, Mlamed J, Scher HI, Cordon-Cardo C (1994) Expression of transforming growth factor α and the epidermal growth factor receptor in human prostate tissues. *J Urol* 152:2120-2124.
30. Scher HI, Sarkis A, Reuter V, Cohen D, Netto G, Petrylak D, Lianes P, Fuks Z, Mendelsohn J, Cordon-Cardo C (1995) Changing pattern of expression of the epidermal growth factor receptor and transforming growth factor- α in the progression of prostatic neoplasms. *Clin Cancer Res* 1:545-550.
31. Sandgren EP, Luetke NC, Palmiter RD, Brinster RL, Lee DC (1990) Overexpression of TGF- α in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. *Cell* 61:1121-1136.
32. Steiner MS (1993) Role of peptide growth factors in the prostate. A review. *Urology* 42:99-110.
33. DiMarco E, Pierce JH, Fleming TP, Kraus MH, Molloy CJ, Aaronson SA, Di Fiore PP (1989) Autocrine interaction between TGF α and the EGF-receptor: Quantitative requirements for the induction of the malignant phenotype. *Oncogene* 4:831-838.
34. Davies DE, Chamberlin SG (1996) Targeting the epidermal growth factor receptor for therapy of carcinomas. *Biochem Pharmacol* 51:1101-1110.
35. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72:248-254.



PubMed

Nucleotide

Protein

Genome

Structure

PMC

Taxonomy

OMIM

Search

PubMed

for

Limits

Preview/Index

History

Clipboard

Details

About Entrez

Abstract

Show:

20

Sort

Text

Text Version

Entrez PubMed

Overview

Help | FAQ

Tutorial

New/Noteworthy

E-Utilities

PubMed Services

Journals Database

MeSH Browser

Single Citation Matcher

Batch Citation Matcher

Clinical Queries

LinkOut

Cubby

Related Resources

Order Documents

NLM Gateway

TOXNET

Consumer Health

Clinical Alerts

ClinicalTrials.gov

PubMed Central

Privacy Policy

1: Reprod Fertil Dev 1995;7(6):1465-70

Related Articles, Links

Role of epidermal growth factor (EGF) and its receptor in the development of the human placenta.

Maruo T, Matsuo H, Otani T, Mochizuki M.

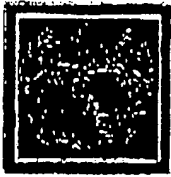
Department of Obstetrics and Gynaecology, Kobe University School of Medicine, Japan.

To elucidate the role of EGF in human placental development, effects of EGF on the proliferation and differentiation of trophoblasts were investigated. Explants of trophoblastic tissues obtained from 4-5 week or 6-12 week placentas were, respectively, cultured with or without EGF, in the presence or absence of triiodo-L-thyronine (T3) in a serum-free condition. The proliferative activity was examined by immunocytochemical staining with an antibody Ki-67, and the differentiated function was assessed by the ability to secrete human chorionic gonadotrophin (hCG) and human placental lactogen (hPL). In 4-5 week placentas, EGF and EGF receptor were localized in cytotrophoblast (C-cell), and EGF augmented the proliferation of C-cell without affecting the ability to secrete hCG and hPL. In contrast, in 6-12 week placentas, EGF and EGF receptor were localized in syncytiotrophoblast (S-cell), and EGF stimulated the secretion of hCG and hPL without affecting the proliferation of C-cell. In situ hybridization with c-erb B probe revealed that c-erb B mRNA is expressed in the S-cell after 6 weeks' gestation. Column chromatography of the serum-free media obtained by 5-day culture of early placental tissues resulted in the elution of immunoreactive EGF. The addition of T3 (10^{-8} mol L⁻¹) resulted in increased secretion of immunoreactive EGF by placental explants. These findings suggest that EGF acts as an autocrine factor in regulating early placental growth and function in synergy with thyroid hormone.

Publication Types:

- Review
- Review, Tutorial

PMID: 8743148 [PubMed - indexed for MEDLINE]



[Frontiers in Bioscience 4, d87-101, January 15, 1999]



Received: 9/30/98
Accepted: 12/18/98

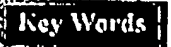
Send
correspondence to:

Daniel Ménard,
Ph.D.,
Professor of Cell
Biology,
Département
d'anatomie et de
biologie cellulaire,
Faculté de médecine,
Université de
Sherbrooke,
Sherbrooke (Québec)
Canada J1H 5N4

Tel.: 819-564-
5278.

Fax: 819-564-
5320.

E-mail:
dménard@courrier
usherb.ca



EGF receptor; EGF-
binding; Human fetal
gut; Intestinal
mucosa; Gastric
mucosa; Cell
proliferation; Brush
border hydrolases;
Gastric enzymes



Copyright © Frontiers

ONTOGENY OF EGF RECEPTORS IN THE HUMAN GUT

Pierre Chailler and Daniel Ménard

Groupe du CRM sur le Développement Fonctionnel et la Physiopathologie du Tube Digestif, Département d'anatomie et de biologie cellulaire, Faculté de médecine, Université de Sherbrooke, Sherbrooke (Québec) Canada

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Identification and biochemistry of EGF receptor
 - 3.1. Gene and protein structure
 - 3.2. Mechanism of activation
 - 3.3. General distribution
 - 3.4. Ligands
4. Expression in human fetal gut segments
 - 4.1. Small intestine
 - 4.2. Colon
 - 4.3. Stomach
5. Comparison with animal models
6. Integration and perspectives
7. Acknowledgements
8. References

1. ABSTRACT

Epidermal growth factor and related substances mediate their effects on epithelial cells through binding to high-affinity receptors (EGF-R) at their basolateral surface and it is hypothesized that this growth factor system play a major role in gut morphogenesis and maintenance. The current review emphasizes on analyzing the expression and the biochemical characteristics of EGF-R in human fetal gut segments and correlating the biological actions of EGF-R ligands. They appear to be primarily involved in the local regulation of epithelial cell proliferation in which EGF-R are abundant. Alternatively, EGF-R ligands exert some precocious maturative effects by increasing intestinal lactase activity and decreasing brush border hydrolases in colon while they down modulate the expression of segment-specific markers of terminal differentiation such as sucrase, trehalase and glucoamylase in the intestine and chief cell lipase in the stomach. Such effects are consistent with the identification of receptors at the surface of all epithelial cell types, illustrating the modulatory role of EGF on differentiated gut epithelial cells. Comparison with animal models illustrates similar biochemical properties of

REVIEW, 1999

receptors and underlines physiological aspects specific to human gut development. The relevance for ligand heterogeneity is also discussed and tentatively associated with different delivery pathways or physiological responses.

2. INTRODUCTION

Epidermal growth factor or EGF is one of the best characterized growth factors that was initially isolated by Stanley Cohen as a contaminating material in nerve growth factor preparations promoting the growth of embryonic neurons (1,2). Early on, it was found to be abundant in some body fluids i.e. saliva, serum, milk and urine (3-6). Accordingly, radioimmunoassay analysis in conjunction with immunohistochemical studies demonstrated that the submaxillary salivary glands, duodenal Brunner's glands, mammary glands and metanephric kidneys represented major sources of the EGF peptide (7-9). EGF-related material (transforming growth factor-alpha or TGFalpha) was also extracted from the amniotic fluid (0.25-4.3 ng/ml; refs 9-11) and found to be produced by extraembryonic membranes (trophoectoderm, placenta) in animals and humans (12-15) although a fraction of this material is likely to derive from developing kidneys (9). Taken altogether, these findings lead to the hypothesis that EGF or related forms released from these sources could be involved in embryonic development and gut morphogenesis or the maintenance of gut tissues in the adult. A major breakthrough was then achieved when EGF mRNA transcripts and EGF-like substance/ activity were detected in lower amounts in various tissues (16-18). Thus, it was proposed that the growth factor could act *in vivo* as a local paracrine factor in addition to its role as a long-distance acting hormone.

Concerning the characterization of the biological action of EGF in tissues, pioneering studies (19,20) using subcutaneous injection protocols in neonatal mice helped to recognize the maturing epidermis and its appendages as important targets of EGF action. It was initially observed that the factor accelerated eyelid opening and incisor eruption (thus accounting for the 'tooth lid factor' denomination used in the early 1960's). Ulterior experiments showed that EGF was also able to stimulate hair growth and epidermal cell proliferation and keratinization (21). The mitogenic effect of EGF was then rigourously studied *in vitro* on fibroblasts (22-25) where the predicted presence of EGF surface receptors was verified for the first time using iodinated (125 I)EGF binding techniques (24,26). As will be discussed further, the presence of surface binding sites was also demonstrated in mesodermal and endodermal derivatives, especially in gut epithelial compartments, and the gene encoding the EGF-R protein in rodents and man was successfully cloned and sequenced (see reviews 27-29).

In 1983, an oncogenic protein, termed v-*erbB1*, was identified as a product of avian erythroblastosis virus (30) which causes the abnormal presence of immature blood cells (erythroblasts) into systemic circulation in chicken. Viral *erbB1* in fact represents a truncated and constitutively-active form of the EGF-R (30); a cellular homolog oncogene named c-*erbB1* was identified in embryonic and tumor cells in rodents (31), and then found to be identical to the mouse EGF-R. Such discoveries and biochemical/immunological data from pathological studies permitted to establish a functional relationship between the expression of EGF-R and the

initiation of cancer, as proposed for the gut in particular, where the relative abundance of EGF-R serves as a prognostic factor in esophageal, gastric and colon tumorigenesis (32-34). Enhanced expression of EGF-R may also be a marker for the increased risk of colon cancer in inflammatory bowel disease (35).

In the following years, the expression pattern, the mechanism of activation, the recycling through the endocytic pathway as well as the involvement of EGF-R in the biological action of EGF were extensively studied in normal tissues and cultured cells originating from animals and man. As exemplified in basic reviews (2,36-40) integrating all aspects of EGF/EGF-R biochemistry and physiology, the expression and function of this growth factor system seem to be primarily associated with the involvement of EGF as a competence growth factor stimulating the survival, the migration and the mitosis of stem cells and pre-differentiated cells, including those of the gastrointestinal tract giving rise to mature epithelial cells. The more recent development of gene knock-out and promoter engineering strategies which enable the generation of mouse lineages carrying either EGF/TGFalpha/EGF-R genes inactivated by defective mutations or genes which expression is forced by an active promoter (mutations integrated in the whole organism or targeted to specific organs) indeed supports the latter concept. Overexpression of the TGFalpha transgene (41-46) primarily alters epithelial proliferative compartments and ultimately leads to a disequilibrium between pre-differentiated and differentiated cell populations: hyperplasia and rarefaction of differentiated phenotypes. Alternatively, null mutations of TGFalpha/EGF-R genes (47-50) reveal that they exert a complex function since their deficiencies not only modify cell proliferation but perturb a wide range of developmental activities (branching morphogenesis, epithelial cell differentiation or maturation, skin architecture). In accordance with the latter observations, other pioneering studies suggest that EGF itself or EGF acting in synergism with other growth factors plays a role in the differentiation of specific cell species such as keratinocytes (as mentioned above; ref.19-21), palatal cells (51), surfactant-secreting pneumocytes (52,53), cervical cells (54), nasal epithelial cells (55), ameloblasts/odontoblasts (56), trophoblasts (57) as well as enterocytes (58-60). These observations jointly reinforces the physiological importance of EGF and EGF-related molecules as cell inducers and stresses the complexity of regulatory processes involved in organogenesis and tissue maintenance. In this context, a growth factor system may play an epigenetic role by stimulating distinct functions in different cell types at specific developmental stages when the ordered sequence of survival, proliferation and differentiation is normally progressing. The following sections describe the general properties of the EGF-R as well as its expression pattern and biological significance in specific segments of the human fetal gastrointestinal tract.

3. IDENTIFICATION AND BIOCHEMISTRY OF EGF RECEPTOR

3.1. Gene and protein structure

The nucleotide sequence of the EGF-R, which is termed HER-1 in human (61), predicts a 1186-1210 aa backbone with a molecular weight of 134-135 kilodaltons (kD)(see reviews 36,38). The mature polypeptide is glycosylated at 11-12 asparagine residues, its molecular mass is estimated to ~170 kilodaltons in various tissues and it is characterized by intrinsic tyrosine kinase activity (62-65). In addition, the

extracellular region contains 51 cysteine residues which are concentrated in two domains that cooperate to form a high affinity EGF binding site (66,67; see figure 1). *Drosophila* and *Caenorhabditis* EGF-R homologs contain the same functional domains (68,69) thus revealing a high degree of evolutionary conservation. Although a single human EGF-R gene exists, two spliced variants were revealed in A431 cells (64,70,71) which served in many laboratories as a model for studying EGF-R expression and subcellular trafficking. The physiological significance of this process remains uncertain however since this epidermoid carcinoma cell line produces aberrant forms of the receptor which are absent in nonmalignant tissues (64,72,73). Only a few data concerning the possible existence of alternative splicing mechanism in human carcinomas (71) and developing kidney (74) were obtained in the past. Presumably, this subject has not retained much attention because the respective contributions of 10- and 5.6-kb transcripts for protein expression have not been examined. Nonetheless the recent discovery of a new spliced mRNA species in human placenta with a lower number of base pairs (75) may lead the way to a new comprehension of post-transcriptional regulatory processes controlling the tertiary conformation and function of the EGF-R.

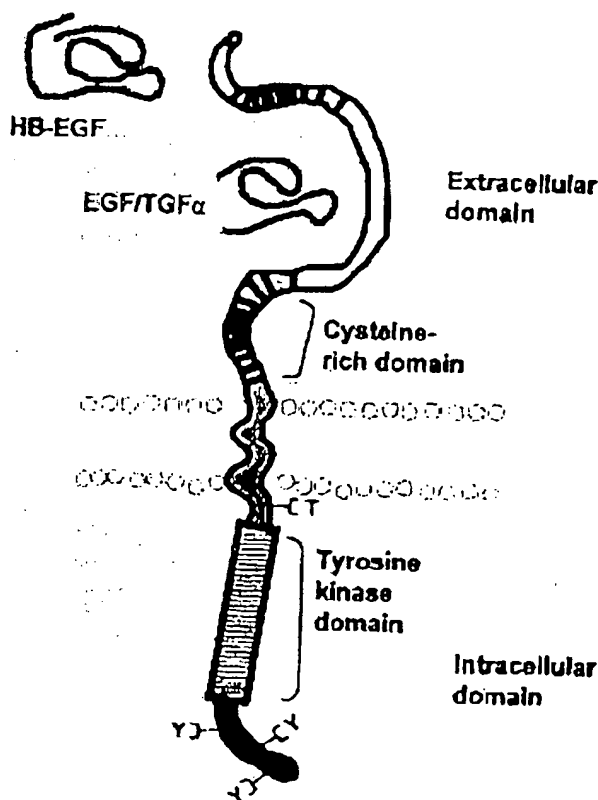


Figure 1. Tertiary structure and consensus motifs of the EGF-R and its common ligands, EGF, TGFα and HB-EGF. The extracellular portion of the receptor contains two domains enriched in cysteine residues (black bars). The intracellular portion contains 1) a catalytic domain with tyrosine kinase activity and 2) several phosphorylation sites at threonine (T) and tyrosine (Y) residues.

3.2. Mechanism of activation

Early studies have demonstrated that EGF binding to the extracellular domain of EGF-R stimulates its tyrosine kinase activity causing increased phosphorylation of protein substrates as well as autophosphorylation of tyrosine residues near the C-terminus region (76). Moreover, threonine phosphorylation of the EGF-R by protein kinase C reduces its binding affinity and negatively regulates tyrosine kinase activity (77). Indeed the functionality of EGF-R varies according to the cell status; high- and low-affinity binding sites were identified in many cell types (78,79) that could be the result of post-translational modifications (phosphorylation, interaction with other proteins)(80,81).

Biochemical data showed in the past that an orderly sequence of receptor dimerization, clustering and internalization is normally activated following EGF binding (82) allowing the cellular response and the endocytic recycling of EGF/EGF-R complexes, as for many receptor systems. In fact, the physical aggregation of multiple EGF/EGF-R complexes at the level of the plasma membrane appears to be required for subsequent activation of tyrosine kinase activity (83,84). Numerous studies have demonstrated over the years that the latter process leads 1) to activation of intracellular effectors associated to EGF-R and which are organized into multimeric complexes (src, ras, phospholipase C- γ , phosphatidylinositol 3-kinase, etc.) and 2) to various nuclear events required for mitogenesis, transformation and cell differentiation (reviewed in 85,86). Recent data even suggest that the receptor aggregation process as well as the optimal phosphorylation of associated effectors are operated through a cooperation with the integrin/matrix apparatus (87). This assumption remains to be verified specifically for the EGF/EGF-R system in the gut.

3.3. General distribution

Putative EGF-R were initially identified and characterized using ^{125}I -EGF binding techniques in a wide range of cell types. Radioautographic data soon established that they are preferentially localized on the plasma membrane of epithelial cells, and at their basolateral pole (reviews 36-40). Moreover their number is high in proliferative cells, neoplastic cells and carcinoma cell lines (27,88). In A431 cells for example, EGF-R are overexpressed by 20 to 50-fold compared to normal tissues (27,64,72). Using the same strategy, receptors were successfully detected in embryonic/fetal and extraembryonic tissues of rodents (36,38,40) where they generally increase in number during gestation while their affinity for the ligand EGF somewhat decreases (89). Also, when fetal organs are explanted and cultured with EGF, the incorporation of (^3H)thymidine into DNA is usually stimulated (90).

Then with the advance of immunological techniques, anti-EGF-R antibodies were produced that helped to confirm the precise localization of the protein at the tissular and subcellular levels. The general distribution of EGF-R in human fetal tissues (91-93) was found to be quite similar, as expected. In comparison with rodents however, the number of receptors tend to decrease before birth probably reflecting the more advanced degree of organ maturation that is achieved *in utero*. One interesting new discovery made on cultured isolated cells pertains to the concentration of EGF-R and associated effectors of signal transduction into adhesion plaques or cytoskeleton-membrane focal adhesions (94,95).

3.4. Ligands

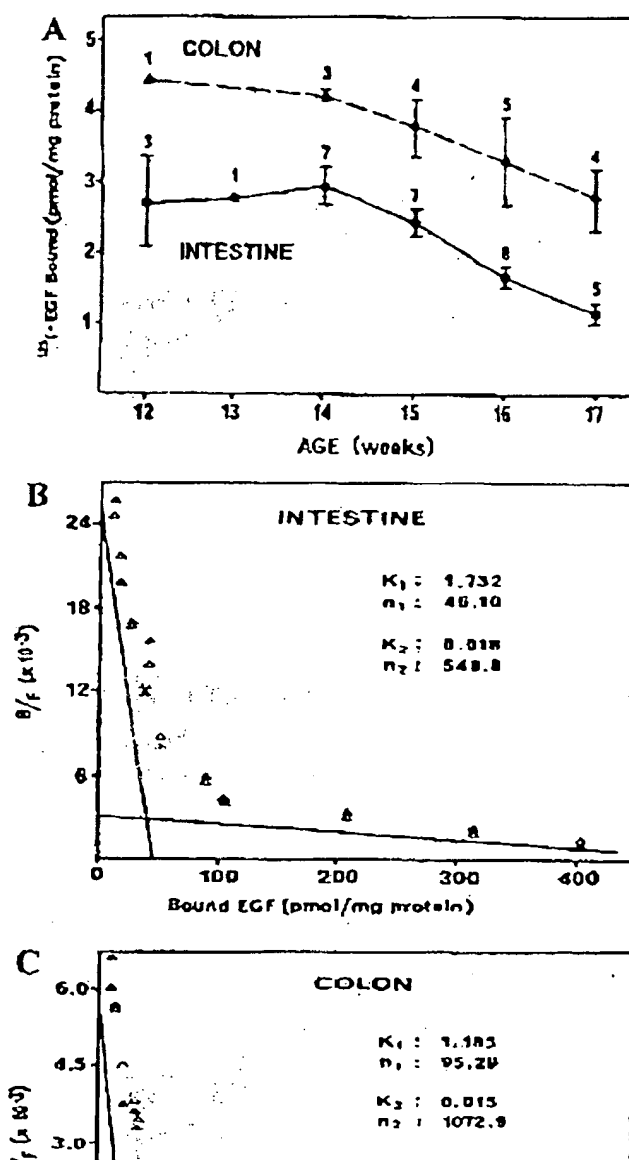
EGF is a 53 amino acid polypeptide, with a molecular weight of 6045 daltons, which possesses three intrachain disulfide bonds. It is the prototypical member of a family of growth factors that also includes human pancreatic secretory trypsin inhibitor (96), transforming growth factor- α (TGF α)(97,98), vaccinia growth factor (99,100), schwannoma-derived growth factor (SDGF) or amphiregulin (AR) (101,102), betacellulin (103), cripto-1 (CR-1)(104), heparin-binding EGF-like factor (HB-EGF)(105), the *neu* differentiation factor (NDF)(106), the heregulins (107), and two homeotic gene products, the *drosophila* Notch protein (108) and the lin-12 protein of the nematode *Caenorhabditis elegans* (109). EGF-related molecules derive from large membrane-bound precursors which are released by proteolytic cleavage (110). PreproEGF, for example, comprises 1217 aa, seven EGF-like repeats in addition to the mature EGF coding region (111,112), and a portion of the molecule exhibits limited homology with the low density lipoprotein (LDL) receptor, suggesting that the EGF precursor and the LDL receptor descended from a common ancestral transmembrane protein (113,114). TGF α derives from a shorter 160 aa precursor (115) and it exhibits 33-44% sequence homology with mouse or human EGF (12 residues are strictly conserved). Of interest, its discovery in the culture medium of established cell lines contributed to elaborate the autocrine hypothesis of cell growth control (116,117). In comparison, HB-EGF is characterized by a longer N-terminal arm with a specific structural motif that confers sensitivity to sulfated glycosaminoglycans and proteoglycans (118). All three peptides represent potent agonists of the EGF-R in several normal (non transformed) tissues and cell lines.

4. EXPRESSION IN HUMAN FETAL GUT SEGMENTS

4.1. Small intestine

The presence of EGF-R in human fetal small intestine was demonstrated for the first time in 1988 by binding measurements (119). In this study, classical ^{125}I -EGF binding procedures were performed at 22° C for 45 minutes on epithelial cell preparations that were obtained by manual shaking (in EDTA/NaCl solution) of everted segments of small intestine. For Scatchard analysis, cells were incubated with the iodinated ligand plus increasing concentrations of unlabelled EGF (10^{-11} to 10^{-7} M) and non-linear plots were analyzed according to the two-site model. The pattern of ^{125}I -EGF binding between 12 and 17 weeks (postfertilization) shows that binding was significantly higher (2.5-fold) in 12 to 14-week fetuses than in older fetuses, as illustrated in figure 2. In percentage terms, specific binding in younger fetuses represented more than 16.5% of labeled EGF/mg cell protein whereas 17-week-old specimens bound less than 6%. Low- and high-affinity binding sites were identified with association constants of $K_1 = 1.90 \pm 0.45 \times 10^{-9} \text{M}^{-1}$ and $K_2 = 0.033 \pm 0.016 \times 10^{-9} \text{M}^{-1}$ respectively. This binding was also specific; no cross-competition for EGF binding sites was observed with insulin, ACTH, thyroxine, hydrocortisone, IGF-1, IGF-2 or dexamethasone when they were added in excess concentration together with EGF. Quantitative autoradiography of ^{125}I -EGF binding (120) reveals

extensive accumulation in undifferentiated cells of the crypt and at the base of the villus, as well as in the inner circular layer of the muscularis externa bordering the submucosa (some labeling was detected in mesenchymal and vascular elements of the lamina propria). A gradient of silver grain density was clearly established along the crypt-villus axis towards the regions of high proliferative activity. Epithelial cells in the deep portion of the crypt showed the highest density (9.2 grains/ μm^2), which gradually decreased in the upper crypt (6.5) and the lower villus (3.9), with very little labeling in the upper third of the villus (0.4). The cellular distribution of silver grains in epithelial cells of the lower villus revealed a polarization of labeling in the basolateral infranuclear region (6.0 grains/ μm^2 versus 0.7 in the supranuclear/brush border compartment). The last observations support the hypothesis that EGF would access to its receptors on epithelial cells from the serosal side, not from the gut lumen, under normal non-pathological conditions. Confirming this assumption, the same study (120) demonstrated that labeled EGF could not access to its receptors when infused into the lumen of jejunal segments, either at 22° C or 4° C.



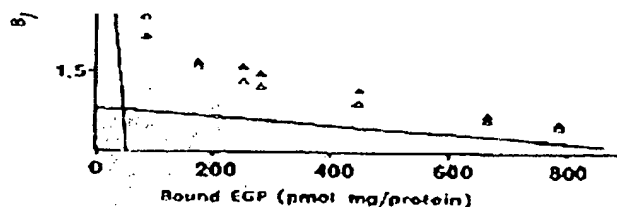


Figure 2. Developmental pattern (A) and Scatchard analyses (B,C) of iodinated EGF binding to epithelial cells isolated from human fetal small intestine and colon. Indices in graph A represent the number of specimens for each age (adapted from ref 119).

Of particular interest, the small intestine undergoes extensive morphogenesis between 8 and 12 weeks of gestation with the onset of villus and crypt formation and subsequent transformation from pseudostratified columnar to simple columnar epithelium (121). Labeling indices within this epithelium are also at their highest value between 8 and 10 weeks (approximately 26-30%) and decrease markedly during the next 4-6 weeks (122). It is thus during this critical period of morphogenesis and remodeling that the intestinal epithelium exhibits its highest EGF binding capabilities. Moreover, Poulsen and co-workers (9) have demonstrated the presence of immunoreactive EGF in intestinal Paneth cells of the 20-week-old fetus suggesting that this cell type represents an endogenous source of the peptide. Paneth cells normally appears at 11-12 weeks (122) which again correlates with the high number of EGF-R at this early age. The possibility that EGF from Paneth cells may influence the neighbouring undifferentiated cells has yet to be explored. In subsequent studies using the immunohistochemistry approach (123,124), the presence of EGF-R at the basolateral cellular pole, their consistent absence in brush border preparations and their rarefication with increasing age were verified in intestinal epithelium of fetuses, children and adults.

Two colon carcinoma cell lines, namely Caco-2 and HT-29, exhibit human enterocyte-like features in culture and are commonly used as *in vitro* models to study the role of putative regulators. The capacity of these cells to bind EGF has been demonstrated (125) as well as the involvement of an EGF-like factor in their autonomous growth (126,127) and migration behavior over laminin substrate (128). Recent data indeed show that antisense EGF-R expression has an antiproliferative effect on HT-29 cells (129). While both Caco-2 and HT-29 cells express a minor pool of EGF-R at their apical surface, only basolateral membrane stimulation with EGF increased tyrosine kinase activity and enhanced proliferation (130,131), in accordance with the proposed model of EGF action on epithelial cells. EGF not only stimulates DNA synthesis and proliferation of Caco-2 cells but reduces sucrase activity markedly, by affecting its processing in the endoplasmic reticulum and mRNA synthesis (132). The same study reveals that EGF maintains Caco-2 cells in a poorly-differentiated phenotype thus suggesting that the growth factor would act as a mitogen and a repressor of terminal differentiation. Interestingly, ulterior experiments showed that parental HT-29 and sublines lose their proliferative response to EGF ligands as they spontaneously differentiate in culture (133): they are mitogenic for undifferentiated cells but inhibit the growth of more differentiated cells. Changes in the signaling machinery rather than the modulation of EGF-R

expression appear to be involved in this process.

The study of EGF-R expression and ligand activity in primary cultures of human small intestinal cells still awaits the development of a suitable experimental system. Nonetheless, the biological effects of EGF have been carefully examined in organ cultures of fetal jejunum (11 to 14 week of gestation) (134). While the addition of exogenous EGF did not modify the morphology of intestinal explants, lactase activity was significantly increased and the rise in sucrase, trehalase and glucoamylase activities that normally occurs during culture was repressed in the presence of increasing concentrations of EGF. DNA synthesis and labeling index dropped drastically (within 24 hours of culture) in this model, thus recalling the response of differentiated cultured cell lines. These results clearly suggest that the influence of EGF on the regulation of small intestinal epithelium development depends upon the differentiation status of target cells.

4.2. Colon

Binding of ^{125}I -EGF to colonic epithelial cells isolated from 12 to 17-week fetuses was assessed using the same methodology and optimal conditions as for intestinal epithelial cells (119; figure 2). Compared to the small intestine, specific binding in the colon was substantially higher (1.4 to 2.4-times) at all ages and also decreased with advancing age (figure 2). High-affinity ($K_1 = 1.78 \pm 0.83 \times 10^{-9} \text{ M}^{-1}$) and low-affinity ($K_2 = 0.014 \pm 0.005 \times 10^{-9} \text{ M}^{-1}$) binding sites were similarly demonstrated by Scatchard plot. Their cellular distribution in smooth muscle fibers as well as in developing villi and crypts of the fetal colon was identical to that found in small intestine, being concentrated in regions of high proliferative activity and tritiated thymidine uptake while being absent from the brush border of villus cells (120).

The overall pattern of EGF binding in the human fetal colon is notheworthy since colonic epithelial cells exhibited even higher binding than their intestinal counterpart. This difference can be attributed partly to a greater concentration of EGF-R at the cell surface. Moreover, since the onset of villus formation and epithelial differentiation in the developing colon occurs 3-4 weeks after initiation of morphogenesis in the small intestine, there may exist a relation between the fact that levels of EGF binding in the colon at 17 weeks are equivalent to peak levels observed in the 12-14 week-old intestine (figure 2). It is also known that the expression of intestinal-type markers are down-regulated and that fetal colonic villi are transformed into adult-type colonic crypts before birth. Whether the expression and binding kinetics of EGF-R are modified during this ontogenic process, when epithelial cell proliferation contributes to tissue remodeling, remains to be determined.

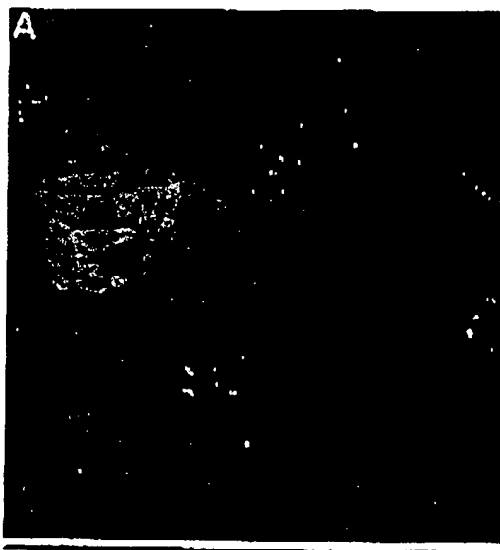
As mentioned previously for the small intestine, the influence of EGF was studied in organ cultures of human fetal colon (135) where it did not alter the overall morphology of explants. Biochemical data, however, suggest that EGF may participate in the maturation process (loss of villus markers) since it caused a decrease of DNA synthesis rates and brush border sucrase, maltase and alkaline phosphatase activity levels. Illustrating the significance and the specificity of EGF

action, hydrocortisone generated no effect in this model (135).

4.3. Stomach

The biological relevance of EGF action at the level of the stomach has been demonstrated early on. The molecule called urogastrone, an inhibitor of gastric acid secretion produced by the human kidney and present in urine, was proven to be identical to the mature EGF peptide (136). The presence of EGF-R in the adult gastric mucosa was also verified and their increased expression in foveolar and surface epithelial compartments (compared to the base of glands) supports a pivotal role for this growth factor system in the maintenance of gastric mucosal integrity (protection, healing)(137-139). However the developmental aspects of EGF-R physiology in the human fetal stomach were only studied recently.

The presence of the EGF-R immunoreactive protein was first suggested in a study analyzing its widespread expression in digestive tract epithelia and pancreatic tissues (123). Using indirect immunofluorescence and autoradiographic localization of ^{125}I -EGF binding sites, it was then demonstrated that the pattern of EGF-R expression in developing stomach significantly differed from the adult situation (140). In fact, EGF-R were detected as soon as 10-11 weeks of gestation when the gastric mucosa is lined by a stratified undifferentiated epithelium. At subsequent stages (12 to 20 weeks; figure 3), all epithelial cell types of the developing pit/gland structure (undifferentiated, mucous, endocrine, parietal and chief cells) were immunoreactive and the three major functional compartments e.g. the surface epithelium, the pit/neck region and the gland contained comparable numbers of binding sites. Thus, when compared to the adult stomach, it seems plausible that EGF-R and its ligands exert a broad range of developmental activities in the fetal organ due to their ubiquitous expression in all gastric epithelial cell species. As expected also, the cellular distribution of EGF binding was concentrated in the infranuclear region of epithelial cells (140). Reflecting the highly polarized localization of EGF-R and, possibly, the early compartmentalization of EGF-R expression in the pit/gland unit, an intensive immunostaining was seen at the basolateral pole of surface mucous cells in 20-week specimens (figure 3).



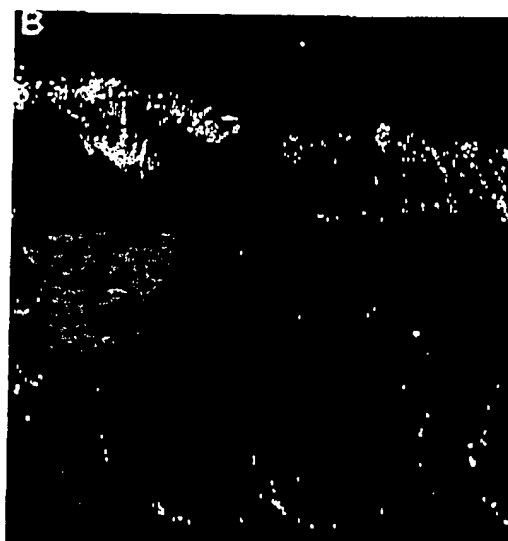


Figure 3. Localization of immunoreactive EGF-R protein in human fetal stomach mucosa at 12 (A) and 20 (B) weeks of gestation. Apical staining on surface cells represents non specific binding of antibody to mucus (from ref.140).

In the past, only a few studies were aimed at studying the role of the EGF/TGF α /EGF-R system in cultured human gastric tissues. One report (141) helped to confirm that EGF-R are present on epithelial cells isolated from adult stomach and showed that exogenous EGF stimulates the release of an angiogenic growth factor. Results obtained with human fetal gastric tissue maintained in organ culture (140) demonstrated that EGF regulates the proliferative and differentiative functions of the developing epithelium. The factor was able to stimulate DNA synthesis in mitogenically-competent cells and the incorporation of tritiated glucosamine into newly-synthesized glycoproteins (in mucous cells). Moreover, EGF down-regulated lipase activity in this model without affecting pepsin, two digestive enzymes co-localized in human glandular chief cells. The latter observations are consistent with the ubiquitous expression of EGF-R in all epithelial cells of the fetal gastric mucosa and further reinforce the assumption that associated ligands might control a variety of developmental activities contributing to the differentiation and/or the maintenance of gastric epithelial lineages. These results also emphasize the uncoupled regulatory process for lipase and pepsin, and enlighten the role of EGF in the modulation of lipolytic activity of the human gastric mucosa. Recently, it has been shown that EGF exerts its down-regulatory action on gastric lipase at the mRNA level (142).

5. COMPARISON WITH ANIMAL MODELS

On a general basis, the characteristics of EGF receptors in both human fetal intestinal and colonic epithelial cells in terms of binding kinetics, ligand specificity and binding affinities ($K_d = 10^{-10}$ to 10^{-9} M) are typical of those found in rodent intestinal epithelial cells using the same protocols (143-145). As seen for the human, the concentration of EGF-R was higher in mouse developing colon than in small intestine at the same postnatal age (146).

While there is a general agreement about the tissular localization of EGF-R and the growth-promoting effect of EGF-related factors on the adult gastrointestinal tract of several species, discrepancies between humans and rodents have been reported regarding its implication in the functional differentiation of intestinal epithelia during ontogeny (refer to comprehensive reviews 147-149). For example, EGF promotes in organ culture the maturation of the rough endoplasmic reticulum and some brush border enzymes of mouse small intestine at the fetal stage (15-17 days) (150-152) in accordance with its suggested role as an inducer of enterocyte differentiation with a specific and restricted action. In suckling (8 days) animals however, injections of EGF stimulate proliferation in all gut segments, including small intestine, and simultaneously increase all brush border hydrolase activities in a dose-dependent manner (153). Therefore, EGF does not only act as a fetal promoting agent but seems to be one of the few factors involved in re-differentiation of epithelial cells during postnatal adaptation of intestinal function at weaning, a process which is highly coordinated along the entire digestive tract in rodents (147). Discrepancies were even observed between mouse and rat as exogenous EGF triggered limited effects on some brush border enzymic activities (154) or had no influence (155,156) in the latter species. In addition, EGF administered orogastrically but not intraperitoneally induced precocious maturation of intestinal disaccharidase activities in suckling rabbits (157). Many explanations can be put forward to explain these variations such as the amount of EGF used, route of administration, rapid uptake of exogenous EGF by the liver, phase of circadian rhythm, nutritional state, length of the experimental period, injection schedule and developmental stages as well as the possible masking effect of endogenous EGF ligands. Eventhough animal models are necessary for the identification of involved growth factors and characterization of their biological roles, it is clear that effects induced by EGF in rodent small intestine cannot be directly extrapolated to the human gut.

There are several evidences that EGF, TGFalpha and related substances may exert a similar influence on developing stomach of rodents (rat, rabbit, guinea pig) and humans (reviewed in 158). They stimulate epithelial proliferation and mucus synthesis while they act negatively on parietal cell differentiation. Moreover targeted overexpression of TGF-alpha gene in mice causes alterations of the gastric mucosa that resemble Ménétrier's disease (46,159,160), a premalignant disorder characterized by foveolar hyperplasia, hypochlorhydia and increased mucus. Thus it appears that this hormonal system would assume a general and important role in development of the mucigenic gastric lineage. The earlier finding by Dembinski and Johnson (161) that EGF significantly increased weight of the whole stomach and DNA content of the oxyntic glands in unweaned rats but had no effect on pepsinogen synthesis/secretion led to the conclusion that EGF is a candidate inducer of oxyntic mucosal growth which does not participate in the developmental regulation of gastric zymogen expression. Our recent observation that this growth factor specifically influences gastric lipase expression in human chief cells (an enzyme absent in rodents) without affecting pepsinogen (140) stresses again the caution to be taken in directly extrapolating concepts established in animal models to humans.

6. INTEGRATION

In 1976, David Wingate formulated a new global theory of gastrointestinal hormone action, called the eupeptide system (162), which stated that the established model of endocrine function - the concept of endocrine glands secreting at a distance from target tissues - was inappropriate for the context of gut physiology. This theory proposed that local 'paracrine' polypeptides may be no less important and may cooperate with 'endocrine' true hormones or mediate some of their effects in the control of nutrient absorption, smooth muscle contraction and tissue maintenance. One must realized today that the discovery and the study of the EGF/EGF-R system has greatly contributed to the conceptual enlightenment of the former theory. As suggested by the variety of its biological actions and its widespread expression in developing and adult gut segments, this growth factor system seems to be involved in the global and intrinsic control of gut morphogenesis and homeostasis.

The current review emphasized on analyzing the expression of EGF-R in human fetal gut and correlating the biological actions of EGF-R ligands. The data presented reveal the ubiquitous distribution of the receptor in the developing mucosa of gut segments as well as in the various epithelial cell types. In comparison, its expression is known to become more restricted in the corresponding adult tissues. We may therefore propose an important and pleiotropic role for the EGF-R and its ligands in the context of gut epithelium development. Firstly, the growth factor system appears to be involved in the local regulation of epithelial cell proliferation in which EGF-R are abundant. Depending upon the specific segment and its differentiation status, cell proliferation would be stimulated or decreased by agonists of EGF-R; the growth factor acts as a mitogen for gastric explants and as an inducer of precocious maturation in small intestine (lactase activity increased) and colon (intestinal enzymes down-regulated). A second function of this system *in utero* may be to counter-regulate the terminal differentiation of human digestive epithelia; it negatively modulates the expression of segment-specific functional markers such as brush border sucrase, trehalase and glucoamylase in the intestine and chief cell lipase in the stomach. In the latter organ, the relative abundance of EGF-R in surface epithelium and the effect of EGF supplementation in culture also argues for a role in mucus synthesis. In addition, this hormonal system is involved in the normal maintenance of adult gut tissues (plus inhibition of gastric acid secretion) and it is deregulated in cancer (148,149). These facts illustrate that EGF-R and EGF-related factors still exert significant developmental activities in the more mature digestive tract.

According to the literature, multiple ligands for the EGF-R are present in tissues, including fetal organs. What would be the relevance of such a heterogeneity in the context of gut epithelium development and maintenance? Probably to serve in different delivery pathways or in different physiological responses (see figure 4). TGFalpha, for example, likely represents the fetal and paracrine ligand, which is synthesized in proliferative compartments of human fetal gut (123,163,164) and which locally regulates epithelial proliferation/differentiation. This factor activates EGF-R and mitogenesis as potently as EGF *in vitro* (165). It might be of some significance in this context that TGFalpha acts more strongly than EGF on cell proliferation, motility and branching morphogenesis in specific experimental systems (166 plus several studies cited in 148,149) due to differences in ligand

processing, receptor down-regulation and, possibly, binding efficiency. The global expression of both TGF α and cognate EGF-R are simultaneously down-regulated in maturing organs while EGF expression increases at the end of gestation and experiments realized in mouse provided evidences that these developmental changes are under the control of thyroid hormones (167,168). However, expression of the TGF α protein persists in a number of mature tissues and recent data demonstrate that it represents the main form locally produced in proliferative compartments of the adult gastrointestinal tract e.g. intestinal crypts and gastric glands (123,169-171). There are new evidences that HB-EGF, another agonist of EGF-R, might be the autocrine form expressed by epithelial cells in response to environmental factors. It is expressed together with EGF or TGF α in many tissues (172) and it is rapidly up-regulated by EGF-related ligands themselves (173) and by *Helicobacter pylori* in gastric cells (174). Its expression greatly increases in gut-associated carcinomas (175,176). HB-EGF thus represents an immediate early gene candidate. Also at the specific level of the human stomach, HB-EGF appears to be the main form produced by fundic parietal cells and gastrin cells of pyloric glands (177). Concerning EGF, the prototypical growth factor, it likely represents the long-distance acting form released into the gut lumen (a lumone) and one current hypothesis is that it would assume a surveillance role by maintaining the integrity of gut tissues upon mucosal damage (stimulation of epithelial restitution and proliferation)(178,179). It is interesting to remind, in this regard, earlier data from Wright and collaborators (180) showing that ulceration of the mucosal epithelium in the human gastrointestinal tract induced the development of a novel cell lineage that produced neutral mucin and abundant immunoreactive EGF. It is true that EGF secreted into the lumen is processed into smaller and less active forms (181,182) and that its stability is positively influenced by ingestion of food proteins (183,184). In the specific context of gut epithelial development in the human fetus and infant, it should be pointed out that EGF may remain bioactive for long periods in gastric and intestinal fluids due to the immaturity of pancreatic enzymic function (185) and the protective effect of milk proteins (186). However, the biological significance and efficiency of luminal binding on epithelial cells (especially in immature gut), the controversial identification of low- or high-affinity binding sites on apical membranes as well as the involvement of a transcytosis mechanism (serosal towards luminal or luminal towards serosal) remain to be clarified (148,187).

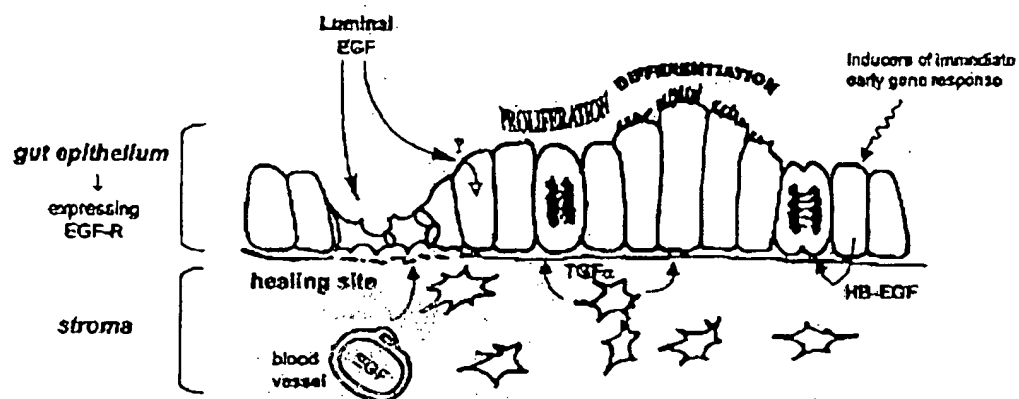


Figure 4. Theoretical model of EGF action on the EGF-R expressing gut epithelium.

EGF-related substances (EGF, TGF α , HB-EGF) derived from various sources would access to EGF-R at sites of epithelial damage or would be released in a paracrine/autocrine fashion at the epithelio-mesenchymal interface to participate in normal regeneration and differentiation.

7. PERSPECTIVES

The overall characteristics of EGF/EGF-R physiology in the developing human gastrointestinal tract do not entirely correlate with those found in animal models. The expression of the receptor or its ligands may also be altered or subjected to abnormal regulatory events in adenocarcinoma cell lines. For these reasons, it will be necessary to establish normal human epithelial cell models in order to further our understanding of the specific developmental actions of this growth factor system and its modulation during successive stages of epithelial differentiation.

Concerning the molecular properties of EGF-R activation and regulation, which are determined by the level of EGF-R expression and modulated by intracellular kinases, it now appears that they depend upon heterodimerization events with EGF-R-related receptors expressed in epithelial cells. HER-2, HER-3 and HER-4 (erbB2, erbB3 and erbB4 in mouse) are known to bind neuregulin-like substances and differential heterodimerization of EGF-R with each partner receptor might account for distinct responses (stimulatory or inhibitory) observed in given cell types (188,189). Interestingly, these EGF-R-related proteins seem to be abundantly expressed in human fetal gut compared to the adult (190,191) and some evidence for their dynamic expression in relation with epithelial morphogenesis was recently reported (192). At the light of experiments exploring MAP kinase activation kinetics in the presence of growth factors alone or growth factors with integrin ligands (87), it is now obvious that EGF-R can also synergize with receptors to extracellular matrix proteins for inducing an optimal response. Confirming this theory, the growth of human colon carcinoma cells was differentially stimulated by TGF α on plastic and collagen substratum while the induced-formation of crypt-like structures in 3-dimensional culture increased the expression of specific integrins (193). Future studies must be oriented to enlighten the intracellular events leading to the biological effects and to characterize the molecular events underlying the synergism between EGF-R and integrin ligands specific to human gastrointestinal epithelial cells.

7. ACKNOWLEDGEMENTS

Daniel Ménard (Ph.D.) is director of the research group on the Functional Development and Physiopathology of the Digestive Tract (*Développement Fonctionnel et Physiopathologie du Tube Digestif*) supported by the Medical Research Council (MRC) of Canada. Pierre Chailier (Ph.D.) is a MRC group research associate. The authors wish to thank Drs Francis Jacot and Claude Poulin of the 'Département de la Santé Communautaire' for their helpful and long-lasting collaboration in providing human fetal specimens.

8. REFERENCES

1. Cohen, S.: Epidermal growth factor (Lecture for the Nobel Foundation 1987,

- Stockholm). *In Vitro Cell Dev Biol* 23, 239-246 (1987)
2. Carpenter, G. & S. Cohen: Epidermal growth factor. *Annu Rev Biochem* 48, 193-216 (1979)
3. Gregory, H. & I.R. Willshire: The isolation of the urogastrones - inhibitors of gastric acid secretion from human urine. *Hoppe Seylers Z Physiol Chem* 356, 1765-1774 (1975)
4. Gregory, H., S. Walsh & C.R. Hopkins: The identification of urogastrone in serum, saliva and gastric juice. *Gastroenterology* 77, 313-318 (1979)
5. Carpenter, G.: Epidermal growth factor is a major growth-promoting agent in human milk. *Science* 210, 198-199 (1980)
6. Beardmore, J.M. & R.C. Richards: Concentrations of epidermal growth factor in mouse milk throughout lactation. *J Endocrinol* 96, 283-292 (1983)
7. Heitz, P.U., M. Kasper, S. Van Noorden, J. M. Polak, H. Gregory & A.G.E. Pearse: Immunohistochemical localisation of urogastrone to human duodenal and submandibular glands. *Gut* 19, 408-413 (1978)
8. Kasselberg, A.G., D.N. Orth, M.E. Gray & M.T. Stahlman: Immunocytochemical localization of human epidermal growth factor/urogastrone in several human tissues. *J Histochem Cytochem* 33, 315-322 (1985)
9. Poulsen, S.S., E. Nexø, P. Skov Olsen, J. Hess & P. Kirkegaard: Immunohistochemical localization of epidermal growth factor in rat and man. *Histochemistry* 85, 389-394 (1986)
10. Barka, T., H. Van der Noen, E.W. Gresik & T. Kerenyi: Immunoreactive epidermal growth factor in human amniotic fluid. *Mt Sinai J Med* 45, 679-684 (1978)
11. D'Souza, S.W., R. Haigh, L. Micklewright, P. Konnai & A. Keys: Amniotic fluid epidermal growth factor and placental weight at term. *Lancet* II, 272-273 (1985)
12. Vaughan, T.J., P.S. James, J.C. Pascall & K.D. Brown: Expression of the genes for TGF α , EGF and the EGF receptor during early pig development. *Development* 116, 663-669 (1992)
13. Shigeta, H., M. Taga, A. Katoh & H. Minaguchi: Ontogenesis and distribution of epidermal growth factor immunoreactivity and binding activity in the mouse fetal and neonatal tissues. *Endocrine J* 40, 641-647 (1993)
14. Chia, C.M., R.M.L. Winston & A.H. Handyside: EGF, TGF- α and EGFR expression in human preimplantation embryos. *Development* 121, 299-307 (1995)
15. Stromberg, K., D.A. Pigott, J.E. Ranchalis & D.R. Twardzik: Human term

- placenta contains transforming growth factors. *Biochem Biophys Res Commun* 106, 354-361 (1982)
16. Byyny, R.L., D.N. Orth & S. Cohen: Radioimmunoassay of epidermal growth factor. *Endocrinology* 90, 1261-1266 (1972)
17. Hirata, Y. & D.N. Orth: Epidermal growth factor (urogastrone) in human tissues. *J Clin Endocrinol Metab* 48, 667-679 (1979)
18. Rall, L.B., J. Scott, G.I. Bell, R.J. Crawford, J.D. Penshow, H.D. Niall & J.P. Coghlan: Mouse prepro-epidermal growth factor synthesis by the kidney and other tissues. *Nature* 313, 228-231 (1985)
19. Cohen, S.: Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J Biol Chem* 237, 1555-1562 (1962)
20. Cohen, S. & G. Carpenter: Human epidermal growth factor: isolation and chemical and biological properties. *Proc Natl Acad Sci USA* 72, 1317-1321 (1975)
21. Cohen, S. & G.A. Elliott: The stimulation of epidermal keratinization by a protein isolated from the submaxillary gland of the mouse. *J Invest Dermatol* 40, 1-5 (1963)
22. Armelin, H.: Pituitary extracts and steroid hormones in the control of 3T3 cell growth. *Proc Natl Acad Sci USA* 70, 2702-2706 (1973)
23. Rose, S.P., R.M. Pruss & H.R. Herschman: Initiation of 3T3 fibroblast cell division by epidermal growth factor. *J Cell Physiol* 86, 593-598 (1975)
24. Hollenberg, M.D. & P. Cuatrecasas: Insulin and epidermal growth factor - human fibroblast receptors related to deoxyribonucleic acid synthesis and amino acid uptake. *J Biol Chem* 250, 3845-3853 (1975)
25. Carpenter, G. & S. Cohen: Human epidermal growth factor and the proliferation of human fibroblasts. *J Cell Physiol* 88, 227-238 (1976)
26. Carpenter, G., K.J. Lembach, M. Morrison & S. Cohen: Characterization of the binding of ^{125}I -labeled epidermal growth factor to human fibroblasts. *J Biol Chem* 250, 4297-4304 (1975)
27. Carpenter, G. & J.G. Zendegei: Epidermal growth factor, its receptor, and related proteins. *Exp Cell Res* 164, 1-10 (1986)
28. Mroczkowski, B. & G. Carpenter: Epidermal growth factor. In: *Hormones, Cell Biology, and Cancer: Perspectives and Potentials*, Alan R. Liss, New York, 207-216 (1988)

29. Adamson, E.D.: Oncogenes in development. *Development* 99, 449-471 (1987)
30. Yamamoto, T., T. Nishida, N. Miyajima, S. Kawai, T. Ooi et al.: The *erbB* gene of avian erythroblastosis virus is a member of the *src* gene family. *Cell* 35, 71-78 (1983)
31. Müller, R.: Differential expression of cellular oncogenes during murine development and in teratocarcinoma cell lines. *Cold Spring Harb Conf Cell Prolif* 10, 451-468 (1983)
32. Yasui, W., H. Sumiyoshi, J. Hata, T. Kameda, A. Ochiai, H. Ito & E. Tahara: Expression of epidermal growth factor receptor in human gastric and colonic carcinomas. *Cancer Res* 48, 137-141 (1988)
33. Ozawa, S., M. Ueda, N. Ando, O. Abe & N. Shimizu: Epidermal growth factor receptors in cancer tissues of esophagus, lung, pancreas, colorectum, breast and stomach. *Jpn J Cancer Res* 79, 1201-1207 (1988)
34. Stemmermann, G., S.C. Heffelfinger, A. Noffsinger, Y.Z. Hui, M.A. Miller & C.M. Fenoglio-Preiser: The molecular biology of esophageal and gastric cancer and their precursors: oncogenes, tumor suppressor genes, and growth factors. *Hum Pathol* 25, 968-981 (1994)
35. Alexander, R.J., A. Panja, E. Kaplan-Liss, L. Mayer & R.F. Raicht: Expression of growth factor receptor-encoded mRNA by colonic epithelial cells is altered in inflammatory bowel disease. *Dig Dis Sci* 40, 485-494 (1995)
36. Adamson, E.D. & A.R. Rees: Epidermal growth factor receptors. *Molec Cell Biochem* 34, 129-152 (1981)
37. Gospodarowicz, D.: Growth factors and their action *in vivo* and *in vitro*. *J Pathol* 141, 201-233 (1983)
38. Gill, G.N., P.J. Bertics & J.B. Santon: Epidermal growth factor and its receptor. *Molec Cell Endocrinol* 51, 169-186 (1987)
39. Burgess, A.: Epidermal growth factor and transforming growth factor alpha. *Brit Med Bull* 45, 401-424 (1989)
40. Adamson, E.D.: Developmental activities of the epidermal growth factor receptor. *Curr Top Dev Biol* 24, 1-55 (1990)
41. Dominey, A.M., X.J. Wang, L.E. King jr, L.B. Nanney, T.A. Gagne, K. Sellheyer, D.S. Bundman, M.A. Longley, J.A. Rothnagel, D.A. Greenhalgh et al.: Targeted overexpression of transforming growth factor alpha in the epidermis of transgenic mice elicits hyperplasia, hyperkeratosis, and spontaneous, squamous papillomas. *Cell Growth Differ* 4, 1071-1082 (1993)

42. Webber, E.M., J.C. Wang, L. Wang, G. Merlino & N. Fausto: Overexpression of transforming growth factor-alpha causes liver enlargement and increased hepatocyte proliferation in transgenic mice. *Am J Pathol* 145, 398-408 (1994)
43. Acra, S.A., N. Bulus, G. Bogatcheva, R.J. Coffey & J.A. Barnard: Increased intestinal epithelial proliferation in metallothioneine-transforming growth factor alpha transgenic mice. *Regul Pept* 74, 105-112 (1998)
44. Sharp, R., M.W. Babyatsky, H. Takagi, S. Tagerud, T.C. Wang, D.E. Bockman, S.J. Brand & G. Merlino: Transforming growth factor alpha disrupts the normal program of cellular differentiation in the gastric mucosa of transgenic mice. *Development* 121, 149-161 (1995)
45. Bockman, D.E., R. Sharp & G. Merlino: Regulation of terminal differentiation of zymogenic cells by transforming growth factor alpha in transgenic mice. *Gastroenterology* 108, 441-454 (1995)
46. Goldenring, J.R., G.S. Ray, C.J. Soroka, J. Smith, I.M. Modlin, K.S. Meise & R.J. Coffey Jr: Overexpression of transforming growth factor-alpha alters differentiation of gastric cell lineages. *Dig Dis Sci* 41, 773-784 (1996)
47. Mann, G.B., K.J. Fowler, A. Gabriel, E.C. Nice, R.L. Williams & A.R. Dunn: Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. *Cell* 23, 249-261 (1993)
48. Helmrath, M.A., C.R. Erwin & B.W. Warner: A defective EGF-receptor in waved-2 mice attenuates intestinal adaptation. *J Surg Res* 69, 76-80 (1997)
49. Hansen, L.A., N. Alexander, M.E. Hogan, J.P. Sundberg, A. Dlugosz, D.W. Threadgill, T. Magnuson & S.H. Yuspa: Genetically null mice reveal a central role for epidermal growth factor receptor in the differentiation of the hair follicle and normal hair development. *Am J Pathol* 150, 1959-1975 (1997)
50. Miettinen, P.J., D. Warburton, D. Bu, J.S. Zhao, J.E. Berger, P. Minoo, T. Koivisto, L. Allen, L. Dobbs, Z. Werb & R. Derynck: Impaired lung branching morphogenesis in the absence of functional EGF receptor. *Dev Biol* 186, 224-236 (1997)
51. Pratt, R.M.: Involvement of hormones and growth factors in the development of the secondary palate. In: *Development in mammals*, vol.4. Ed: Johnson, M.H., Elsevier/North-Holland Biomedical Press, New York, 203 (1980)
52. Catterton, W.Z., M.B. Escobedo, W.R. Sexson, M.E. Gray, H.W. Sundell & M.T. Stahlman: Effect of epidermal growth factor on lung maturation in fetal rabbits. *Pediatr Res* 13, 104-108 (1979)
53. Messmer, T.O., R. Armour & R.W. Holley: Factors influencing the growth of

- alveolar type II epithelial cells isolated from rat lungs. *Exp Cell Res* 142, 417-426 (1982)
54. Stanley, M.A. & Dahlenburg: The effect of cholera toxin and epidermal growth factor on proliferation and maturation *in vitro* of human ectocervical cells. *In Vitro* 20, 144-151 (1985)
55. Wu, K., J. Yankaskas, E. Cheng, M.R. Knowles & R. Boucher: Growth and differentiation of human nasal epithelial cells in culture. *Am Rev Respir Dis* 132, 311-320 (1985)
56. Topham, R.T., D.J. Chiego, V.H. Gattone II, D.A. Hinton & R.M. Klein: The effect of epidermal growth factor on neonatal incisor differentiation in the mouse. *Dev Biol* 124, 532-543 (1987)
57. Morrish, D.W., D. Bhardwaj, L.K. Dabbagh, H. Marusyk & O. Siy: Epidermal growth factor induces differentiation and secretion of human chorionic gonadotropin and placental lactogen in normal human placenta. *J Clin Endocrinol Metab* 65, 1282-1290 (1987)
58. Calvert, R., J.F. Beaulieu & D. Ménard: Epidermal growth factor (EGF) accelerates the maturation of fetal mouse intestinal mucosa in utero. *Experientia* 38, 1096-1097 (1982)
59. Malo, C. & D. Ménard: Influence of epidermal growth factor on the development of suckling mouse intestinal mucosa. *Gastroenterology* 83, 28-35 (1982)
60. Oka, Y., F.K. Ghisham, H.L. Green & D.N. Orth: Effects of mouse epidermal growth factor/urogastrone on the functional maturation of rat intestine. *Endocrinology* 112, 940-944 (1983)
61. Prigent, S.A. & N.R. Lemoine: The type 1 (EGFR-related) family of growth factor receptors and their ligands. *Prog Growth Factor Res* 4, 1-24 (1992)
62. Ushiro, H & S. Cohen: Identification of phosphotyrosine as a product of epidermal growth factor-activated protein kinase in A-431 cell membranes. *J Biol Chem* 255, 8363-8365 (1980)
63. Cohen, S., H. Ushiro, C. Stoschek & M. Chinkers: A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. *J Biol Chem* 257, 1523-1531 (1982)
64. Ullrich, A., L. Coussens, J.S. Hayflick, T.J. Dull, A. Gray, A.W. Tam, J. Lee, Y. Yarden, T.A. Libermann, J. Schlessinger, J. Downward, E.L.V. Mayes, N. Whittle, M. D. Waterfield & P.H. Seeburg: Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309, 418-425 (1984)
65. Hunter, T. & J.A. Copper: Protein-tyrosine kinases. *Annu Rev Biochem* 54, 897-

930 (1985)

66. Weber, W., P.J. Bertics & G.N. Gill: Immunoaffinity purification of the epidermal growth factor receptor. *J Biol Chem* 259, 14631-14636 (1984)
67. Gill, G.N., M.G. Rosenfeld, W.S. Chen, P.J. Bertics & C.S. Lazar: Analysis of functional domains in the epidermal growth factor receptor using site-directed mutagenesis. *Adv Exp Med Biol* 234, 91-103 (1988)
68. Livneh, E., L. Glazer, D. Segal, J. Schlessinger & B.Z. Shilo: The drosophila EGF receptor gene homolog: conservation of both hormone binding and kinase domains. *Cell* 40, 599-607 (1985)
69. Aroian, R.V., M. Koga, J.E. Mendel, Y. Oshima & P.W. Sternberg: The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* 348, 693-699 (1990)
70. Xu, Y.H., S. Ishii, A.J.L. Clark, M. Sullivan, R.K. Wilson, D.P. Ma, B.A. Roe, G.T. Merlino & I. Pastan: Human epidermal growth factor receptor cDNA is homologous to a variety of RNAs overproduced in A431 carcinoma cells. *Nature* 309, 806-810 (1984)
71. Xu, Y.H., N. Richert, S. Ito, G.T. Merlino & I. Pastan: Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines. *Proc Natl Acad Sci USA* 81, 7308-7312 (1984)
72. Merlino, G.T., Y.H. Xu, S. Ishii, A.J.L. Clark, K. Semba, K. Toyoshima, T. Yamamoto & I. Pastan: Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. *Science* 224, 417-419 (1984)
73. Stoscheck, C.M. & L.E. King, Jr.: Functional and structural characteristics of EGF and its receptor and their relationship to transforming proteins. *J Cell Biochem* 31, 135-152 (1986)
74. Goodyer, P.R., J. Fata, L. Mulligan, D. Fisher, R. Fagan, H.J. Guyda & C.G. Goodyer: Expression of transforming growth factor-alpha and epidermal growth factor receptor in human fetal kidneys. *Molec Cell Endocrinol* 77, 199-206 (1991)
75. Ilekis, J.V., B.C. Stark & B. Scoccia: Possible role of variant RNA transcripts in the regulation of epidermal growth factor receptor expression in human placenta. *Mol Reprod Dev* 41, 149-156 (1995)
76. Hunter, T. & J.A. Cooper: Epidermal growth factor induces rapid tyrosine phosphorylation of proteins in A431 human tumor cells. *Cell* 24, 741-752 (1981)
77. Cochet, C., G.N. Gill, J. Meisenhelder, J.A. Cooper & T. Hunter: C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor-stimulated tyrosine protein kinase activity. *J Biol Chem* 259, 2553-

2558 (1984)

78. King, A.C. & P. Cuatrecasas: Resolution of high and low affinity epidermal growth factor receptors. *J Biol Chem* 257, 3053-3060 (1982)
79. Kawamoto, T., J.D. Sato, A. Le, J. Polikoff, G.H. Sato & J. Mendelsohn: Growth stimulation of A431 cells by epidermal growth factor: identification of high-affinity receptors for epidermal growth factor by an anti-receptor monoclonal antibody. *Proc Natl Acad Sci USA* 80, 1337-1341 (1983)
80. Lin, C.R., W.S. Chen, C.S. Lazar, C.D. Carpenter, G.N. Gill, R.M. Evans & M.G. Rosenfeld: Protein kinase C phosphorylation at Thr 654 of the unoccupied EGF receptor and EGF binding regulate functional receptor loss by independent mechanisms. *Cell* 44, 839-848 (1986)
81. Sargent, D.F. & R. Schwyzer: Membrane lipid phase as catalyst for peptide-receptor interaction. *Proc Natl Acad Sci USA* 83, 5774-5778 (1986)
82. Schlessinger, J., Y. Schechter, M.C. Willingham & I. Pastan: Direct visualization of binding, aggregation, and internalization of insulin and epidermal growth factor on living fibroblastic cells. *Proc Natl Acad Sci USA* 75, 2659-2663 (1978)
83. Schechter, Y., L. Hernaez, J. Schlessinger & P. Cuatrecasas: Local aggregation of hormone-receptor complexes is required for activation by epidermal growth factor. *Nature* 278, 835-838 (1979)
84. Schreiber, A.B., T.A. Libermann, I. Lax, Y. Yarden & J. Schlessinger: Biological role of epidermal growth factor-receptor clustering. *J Biol Chem* 258, 846-853 (1983)
85. Schlessinger, J. & A. Ullrich: Growth factor signaling by receptor tyrosine kinases. *Neuron* 9, 383-391 (1992)
86. Hunter, T.: Tyrosine phosphorylation: past, present and future. *Biochem Soc Trans* 24, 307-327 (1996)
87. Miyamoto, S., H. Teramoto, J.S. Gutkind & K.M. Yamada: Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *J Cell Biol* 135, 1633-1642 (1996)
88. Velu, T.J.: Structure, function and transforming potential of the epidermal growth factor receptor. *Mol Cell Endocrinol* 70, 205-216 (1990)
89. Adamson, E.D. & J. Meek: The ontogeny of epidermal growth factor receptors during mouse development. *Dev Biol* 103, 62-71 (1984)
90. Adamson, E.D., M.J. Deller & J.B. Warshaw: Functional EGF receptors on embryonic cells. *Nature* 291, 656-659 (1981)

91. Damjanov, I., B. Mildner & B.B. Knowles: Immunohistochemical localization of the epidermal growth factor receptor in normal human tissues. *Lab Invest* 55, 588-592 (1986)
92. Oliver, A.M.: Epidermal growth factor receptor expression in human foetal tissues is age-dependent. *Br J Cancer* 58, 461-463 (1988)
93. Fukuyama, R. & N. Shimizu: Expression of epidermal growth factor (EGF) and the EGF receptor in human tissues. *J Exp Zool* 258, 336-343 (1991)
94. Landreth, G.E., L.K. Williams & G.D. Rieser: Association of the epidermal growth factor receptor kinase with the detergent-insoluble cytoskeleton of A431 cells. *J Cell Biol* 101, 1341-1350 (1985)
95. van Bergen en Henegouwen, P.M., J.C. den Hartigh, P. Romeyn, A.J. Verkleij & J. Boonstra: The epidermal growth factor receptor is associated with actin filaments. *Exp Cell Res* 199, 90-97 (1992)
96. Hunt, L.T., W.C. Barker & M.O. Dayhoff: Epidermal growth factor: internal duplication and probable relationship to pancreatic secretory trypsin inhibitor. *Biochem Biophys Res Commun* 60, 1020-1028, 1974
97. DeLarco, L.E. & G.J. Todaro: Growth factors from murine sarcoma virus-transformed cells. *Proc Natl Acad Sci USA* 75, 4001-4005 (1978)
98. Massagué, J.: Epidermal growth factor-like transforming growth factor. *J Biol Chem* 258, 13606-13613 (1983)
99. Brown, L.P., D.R. Twardzik, H. Marquardt & G.J. Todaro: Vaccinia virus encodes a polypeptide homologous to epidermal growth factor and transforming growth factor. *Nature* 313, 491-492 (1989)
100. Stroobant, P., R.P. Rice, W.J. Gullick, D.J. Cheng, I.M. Kerr & M.D. Waterfield: Purification and characterization of vaccinia virus growth factor. *Cell* 42, 383-393 (1985)
101. Shoyab, M., G.D. Plowman, V.L. McDonald, J.G. Bradley & G.J. Todaro: Structure and function of human amphiregulin: a member of the epidermal growth factor family. *Science* 243, 1074-1076 (1989)
102. Kimura, H., W.H. Fisher & D. Schubert: Structure, expression and function of a Schwannoma-derived growth factor. *Nature* 348, 257-260 (1990)
103. Sasada, R., Y. Ono, Y. Taniyama, Y. Shing, J. Folkman & K. Igarashi: Cloning and expression of cDNA encoding human betacellulin, a new member of the EGF family. *Biochem Biophys Res Commun* 190, 1173-1179 (1993)
104. Ciccodicola, A., R. Dono, S. Obici, A. Simeone, M. Zollo & M.G. Persico:

- Molecular characterization of a gene of the 'EGF family' expressed in undifferentiated human NTERA2 teratocarcinoma cells. *EMBO J* 8, 1987-1991 (1989)
105. Higashiyama, S., J.A. Abraham, J. Miller, J.C. Fiddes & M. Klagsbrun: A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* 251, 936-939 (1991)
106. Wen, D., E. Peles, R. Cupples, S.V. Suggs, S.S. Bacus, Y. Luo, G. Trail, S. Hu, S.M. Silbiger, R.B. Levy, R.A. Koski, H.S. Lu & Y. Yarden: Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell* 69, 559-572 (1992)
107. Holmes, W.E., M.X. Sliwkowski, R.W. Akita, W.J. Henzel, J. Lee, J.W. Park, D. Yansura, N. Abadi, H. Raab, G.D. Lewis, H.M. Shepard, W.J. Kuang, W.I. Wood, D.V. Goeddel & R.L. Vanden: Identification of heregulin, a specific activator of p185^{erbB2}. *Science* 256, 1205-1210 (1992)
108. Wharton, K.A., K.M. Johansen, T. Xu & S. Artavanis-Tsakonas: Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* 43, 567-581 (1985)
109. Greenwald, I.: lin-12, a nematode homeotic gene, is homologous to a set of mammalian proteins that includes epidermal growth factor. *Cell* 43, 583-590 (1985)
110. Burmeister, M., A. Avivi, J. Schlessinger & H. Soreq: Production of EGF-containing polypeptides in *Xenopus* oocytes microinjected with submaxillary gland mRNA. *EMBO J* 3, 1499-1505 (1983)
111. Doolittle, R.F., D.F. Feng & M.S. Johnson: Computer-based characterization of epidermal growth factor precursor. *Nature* 307, 558-560 (1984)
112. Gray, A., T.J. Dull & A. Ullrich: Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000-molecular weight protein precursor. *Nature* 303, 722-725 (1983)
113. Russell, D.W., W.J. Schneider, T. Yamamoto, K.L. Luskey, M.S. Brown & J.L. Goldstein: Domain map of the LDL receptor: sequence homology with the epidermal growth factor precursor. *Cell* 37, 577-585 (1984)
114. Yamamoto, T., C.G. Davis, M.S. Brown, W.J. Schneider, M.L. Casey, J.L. Goldstein & D.W. Russell: The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell* 39, 27-38 (1984)
115. Derynck, R., A.B. Roberts, M.E. Winkler, E.Y. Chan & D.V. Goeddel: Human transforming growth factor- α : precursor structure and expression in *E.coli*. *Cell* 38, 287-297 (1984)
116. de Larco, J.E. & G.J. Todaro: Growth factors from murine sarcoma virus-

- transformed cells. *Proc Natl Acad Sci USA* 75, 4001-4005 (1978)
117. Todaro, G.J., J.E. deLarco, C. Fryling, P.A. Johnson & M.B. Sporn: Transforming growth factors (TGFs): properties and possible mechanisms of action. *J Supramol Struct Cell Biochem* 15, 287-301 (1981)
118. Cook, P.W., D. Damm, B.L. Garrick, K.M. Wood, C.E. Karkaria, S. Higashiyama, M. Klagsbrun & J.A. Abraham: Carboxyl-terminal truncation of leucine⁷⁶ converts heparin-binding EGF-like factor from a heparin-enhancible to a heparin-suppressible growth factor. *J Cell Physiol* 163, 407-417 (1995)
119. Pothier, P. & D. Ménard: Presence and characteristics of epidermal growth factor receptors in human fetal small intestine and colon. *FEBS Lett* 228, 113-117 (1988)
120. Ménard, D. & P. Pothier: Radioautographic localization of epidermal growth factor receptors in human fetal gut. *Gastroenterology* 101, 640-649 (1991)
121. Moxey, P.C. & J.S. Trier: Specialized cell types in the human fetal small intestine. *Anat Rec* 191, 269-286 (1978)
122. Arsenault, P. & D. Ménard: Cell proliferation in developing human jejunum. *Biol Neonate* 51, 297-304 (1987)
123. Hormi, K. & T. Lehy: Developmental expression of transforming growth factor-alpha and epidermal growth factor receptor proteins in the human pancreas and digestive tract. *Cell Tissue Res* 278, 439-450 (1994)
124. Playford, R.J., A.M. Hanby, S. Gschmeissner, L.P. Peiffer, N.A. Wright & T. McGarrrity: The epidermal growth factor receptor (EGF-R) is present on the basolateral, but not the apical, surface of enterocytes in the human gastrointestinal tract. *Gut* 39, 262-266 (1996)
125. Hidalgo, I.J., A. Kato & R.T. Borchardt: Binding of epidermal growth factor by human colon carcinoma cell (Caco-2) monolayers. *Biochem Biophys Res Commun* 160, 317-324 (1989)
126. Culouscou, J.M., F. Garrouste, M. Remacle-Bonnet, D. Bettetini, J. Marvaldi & G. Pommier: Autocrine secretion of a colorectum-derived growth factor by HT-29 human colon carcinoma cell line. *Int J Cancer* 42, 895-901 (1988)
127. Anzano, M.A., D. Rieman, W. Prichett, D.F. Bowen-Pope & R. Greig: Growth factor production by human colon carcinoma cell lines. *Cancer Res* 49, 2898-2904 (1989)
128. Basson, M.D., I.M. Modlin & J.A. Madri: Human enterocyte (Caco-2) migration is modulated in vitro by extracellular matrix composition and epidermal growth factor. *J Clin Invest* 90, 15-23 (1992)

129. Wang, H.M., S. Rajagopal, S. Chakrabarty: Inhibition of human colon cancer malignant cell behavior and growth by antisens epidermal growth factor receptor expression vector. *Anticancer Res* 18, 2297-2300 (1998)
130. Lehmann, M., M. Remacle-Bonnet, F. Garrouste, J. Luis, C. Rabenandrasana, J. Marvaldi & G. Pommier: Surface distribution of the EGF receptor during differentiation of the human colon carcinoma cell line HT29-D4. *J Recept Res* 14, 319-333 (1994)
131. Bishop, W.P. & J.T. Wen: Regulation of Caco-2 cell proliferation by basolateral membrane epidermal growth factor receptors. *Am J Physiol* 267, G892-G900 (1994)
132. Cross, H.S. & A. Quaroni: Inhibition of sucrase-isomaltase expression by EGF in the human colon adenocarcinoma cells Caco-2. *Am J Physiol* 261, C1173-C1183 (1991)
133. Huang, F., S. Sauma, Z. Yan & E. Friedman: Colon absorptive epithelial cells lose their proliferative response to TGF alpha as they differentiate. *Exp Cell Res* 219, 8-14 (1995)
134. Ménard, D., P. Arsenault & P. Pothier: Biological effects of epidermal growth factor in human fetal jejunum. *Gastroenterology* 94, 656-663 (1988)
135. Ménard, D., L. Corriveau & P. Arsenault: Differential effects of epidermal growth factor and hydrocortisone in human fetal colon. *J Pediatr Gastroenterol Nutr* 10, 13-20 (1990)
136. Gregory, H.: The isolation and structure of urogastrone and its relationship to epidermal growth factor. *Nature* 257, 325-327 (1975)
137. Olsen, P.S., S.S. Poulsen, P. Kirkegaard & E. Nexø: Role of submandibular saliva and epidermal growth factor in gastric cytoprotection. *Gastroenterology* 87, 103-108 (1984)
138. Orsini, B., A. Calabro, S. Milani, C. Grappone, H. Herbst & C. Surrenti: Localization of epidermal growth factor/transforming growth factor-alpha receptor in the human gastric mucosa. *Virchows Archiv A Pathol Anat* 423, 57-63 (1993)
139. Konturek, J.W., T. Brzozowski & S.J. Konturek: Epidermal growth factor in protection, repair, and healing of gastroduodenal mucosa. *J Clin Gastroenterol* 13 (suppl 1), S88-S97 (1991)
140. Tremblay, E., S. Monfils & D. Ménard: Epidermal growth factor influences cell proliferation, glycoproteins, and lipase activity in human fetal stomach. *Gastroenterology* 112, 1188-1196 (1997)
141. Takahashi, M., K. Ogura, S. Maeda, K. Mori, K. Mafune, Y. Mikami, A.

Terano & M. Omata: Promoters of epithelialization induce expression of vascular endothelial growth factor in human gastric epithelial cells in primary culture. *FEBS Lett* 418, 115-118 (1997)

142. Ménard, D. & E. Tremblay: Regulation of human gastric lipase (HGL) expression in organ culture by epidermal growth factor (EGF). [Abstract, AGA meeting] *Gastroenterology* 114, A894 (1998)

143. Gallo-Payet, N. & J.S. Hugon: Epidermal growth factor receptors in isolated adult mouse intestinal cells: studies in vivo and in organ culture. *Endocrinology* 116, 194-201 (1985)

144. Gallo-Payet, N., P. Pothier & J.S. Hugon: Ontogeny of EGF receptors during postnatal development of mouse small intestine. *J Pediatr Gastroenterol Nutr* 6:114-120 (1987)

145. Forgue-Laffitte, M.E., M. Laburthe, M.C. Chamblier, A.J. Moody & G. Rosselin: Demonstration of specific receptors for EGF-urogastrone in isolated rat intestinal epithelial cells. *FEBS Lett* 114, 243-246 (1980)

146. Ménard, D., P. Pothier & N. Gallo-Payet: Epidermal growth factor receptors during postnatal development of the mouse colon. *Endocrinology* 121, 1548-1554 (1987)

147. Ménard, D. & R. Calvert: Fetal and postnatal development of the small and large intestine: patterns and regulation. In: *Growth of the Gastrointestinal tract: gastrointestinal hormones and growth factors*. Eds: Morisset, J. & Solomon T.E., CRC Press, Boca Raton, 159-174 (1991)

148. Podolsky, D.K.: Peptide growth factors in the Gastrointestinal Tract. In: *Physiology of the Gastrointestinal Tract*. Ed: Johnson, L.R., 3rd ed., Raven Press, New York, 129-168 (1994)

149. Karnes, W.E. Jr: Epidermal growth factor and transforming growth factor-alpha. In: *Gut peptides. Biochemistry and Physiology (Comprehensive Endocrinology Serie)*. Eds: Walsh, J.H. & Dockray G.J., Raven Press, New York, 553-586 (1994)

150. Beaulieu, J.F. & R. Calvert: The effect of epidermal growth factor (EGF) on the differentiation of the rough endoplasmic reticulum in fetal mouse small intestine in organ culture. *J Histochem Cytochem* 29, 765-770 (1981)

151. Beaulieu, J.F., D. Ménard & R. Calvert: Influence of epidermal growth factor on the maturation of the fetal mouse duodenum in organ culture. *J Pediatr Gastroenterol Nutr* 4, 476-481 (1985)

152. Conteas, C.N., J.M. DeMorrow & A.P. Majumdar: Effect of epidermal growth factor on growth and maturation of fetal and neonatal rat small intestine in organ culture. *Experientia* 42, 950-952 (1986)

153. Malo, C. & D. Ménard: Influence of epidermal growth factor on the development of suckling mouse intestinal mucosa. *Gastroenterology* 83, 28-35 (1982)
154. Oka, Y., F.K. Ghishan, H.L. Greene & D.N. Orth: Effect of mouse epidermal growth factor/urogastrone on the functional maturation of rat intestine. *Endocrinology* 112, 940-944 (1982)
155. Simon, P.M., M. Keding, F. Raul, J.F. Grenier & K. Haffen: Organ culture of suckling rat intestine: comparative study of various hormones on brush border enzymes. *In Vitro* 18, 339-346 (1982)
156. Majumdar, A.P.N.: Postnatal undernutrition effect of epidermal growth factor on growth and function of the gastrointestinal tract in rats. *J Pediatr Gastroenterol Nutr* 3, 618-625 (1984)
157. O'Loughlin, E.V., M. Chung, M. Hollenberg, J. Hayden, I. Zahavi & D.G. Gall: Effect of epidermal growth factor on ontogeny of the gastrointestinal tract. *Am J Physiol* 249, G674-G678 (1985)
158. Coffey, R.J.Jr, M. Romano & J. Goldenring: Roles of transforming growth factor-alpha in the stomach. *J Clin Gastroenterol* 21(suppl.1), S36-S39 (1995)
159. Dempsey, P.J., J.R. Goldenring, C.J. Soroka, I.M. Modlin, R.W. McClure, C.D. Lind, D.A. Ahlquist, M.R. Pittelkow, D.C. Lee, E.P. Sandgren, D.L. Page & R.J. Coffey: Possible role for transforming growth factor alpha in the pathogenesis of Ménétrier's disease, supportive evidence from humans and transgenic mice. *Gastroenterology* 103, 1950-1963 (1992)
160. Takagi, H., C. Jhappan, R. Sharp & G. Merlino: Hypertrophic gastropathy resembling Menetrier's disease in transgenic mice overexpressing transforming growth factor alpha in the stomach. *J Clin Invest* 90, 1161-1167 (1992)
161. Dembinski, A.B. & L.R. Johnson: Effect of EGF on the development of the rat gastric mucosa. *Endocrinology* 116, 90-94 (1985)
162. Wingate, D.: The eupeptide system: a general theory of gastrointestinal hormones. *The Lancet* 1, 529-532 (1976)
163. Yasui, W., J. Zhong-Qiang, H. Kuniyasu, A. Ayhan, H. Yokozaki, H. Ito & E. Tahara: Expression of transforming growth factor alpha in human tissues: immunohistochemical study and Northern blot analysis. *Virchows Archiv A Pathol Anat* 421, 513-519 (1992)
164. Miettinen, P.J.: Transforming growth factor-alpha and epidermal growth factor expression in human fetal gastrointestinal tract. *Pediatr Res* 33, 481-486 (1993)
165. Todaro, G.J., C. Fryling & J.E. DeLarco: Transforming growth factors produced

- of heparin-binding EGF-like growth factor and related peptides in human gastric carcinoma. *Int J Cancer* 66, 315-321 (1996)
177. Murayama, Y., J.I. Miyagawa, S. Higashiyama, S. Kondo, M. Yabu, K. Isozaki, S. Kanayama, Y. Shinomura, N. Taniguchi & Y. Matsuzawa: Localization of heparin-binding epidermal growth factor-like growth factor in human gastric mucosa. *Gastroenterology* 109, 1051-1059 (1995)
178. Playford, R.J. & N.A. Wright: Why is epidermal growth factor present in the gut lumen? *Gut* 38, 303-305 (1996)
179. Goodlad, R.A. & N.A. Wright: Epidermal growth factor and transforming growth factor-alpha actions on the gut. *Eur J Gastroenterol Hepatol* 7, 928-932 (1995)
180. Wright, N.A., C. Pike & G. Elia: Induction of a novel epidermal growth factor-secreting cell lineage by mucosal ulceration in human gastrointestinal stem cells. *Nature* 343, 82-85 (1990)
181. Britton, J.R., C. George-Nascimento & O. Koldovsky: Luminal hydrolysis of recombinant human epidermal growth factor in the rat gastrointestinal tract: segmental and developmental differences. *Life Sci* 43, 1339-1347 (1988)
182. Playford, R., T. Marchbank, D.P. Calnan, J. Calam, P. Royston, J.J. Batten & H.F. Hansen: Epidermal growth factor is digested to smaller, less active forms in acidic gastric juice. *Gastroenterology* 108, 92-101 (1995)
183. Playford, R.J., A.C. Woodman, P. Clark, P. Watanapa, D. Vesey, P.H. Deprey, R.C.N. Williamson & J. Calam: Effect of luminal growth factor preservation on intestinal growth. *Lancet* 341, 843-848 (1993)
184. Marchbank, T., R.A. Goodlad, C.Y. Lee & R.J. Playford: Luminal growth factor is trophic to the small intestine of parenterally fed rats. *Clin Sci* 89, 117-120 (1995)
185. Rao, R.K., O. Koldovsky, M. Korc, P.F. Pollack, S. Wright & T.P. Davis: Processing and transfer of epidermal growth factor in developing rat jejunum and ileum. *Peptides* 11, 1093-1102 (1990)
186. Rao, R.K., R.D. Baker & S.S. Baker: Bovine milk inhibits proteolytic degradation of epidermal growth factor in human gastric and duodenal lumen. *Peptides* 19, 495-504 (1998)
187. Podolsky, D.K.: Peptide growth factors and regulation of growth in the intestine. In: *Gut Peptides. Biochemistry and Physiology (Comprehensive Endocrinology Serie)*. Eds: Walsh, J.H. & Dockray, G.J., Raven Press, New York, 803-824 (1994)
188. Spivak-Kroizman, T., D. Rotin, D. Pinchasi, A. Ullrich, J. Schlessinger & I.

Lax: Heterodimerization of c-erbB2 with different epidermal growth factor receptor mutants elicits stimulatory or inhibitory responses. *J Biol Chem* 267, 8056-8063 (1992)

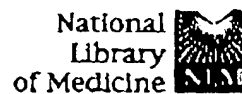
189. Graus-Porta, D., R.R. Beerli, J.M. Daly & N.E. Hynes: ErbB-2, the preferred heterodimerization partner of all erbB receptors, is a mediator of lateral signaling. *EMBO J* 16, 1647-1655 (1997)

190. Mori, S., T. Akiyama, Y. Yamada, Y. Morishita, I. Sugawara, K. Toyoshima & T. Yamamoto: C-erbB-2 gene product, a membrane protein commonly expressed on human fetal epithelial cells. *Lab Invest* 61, 93-97 (1989)

191. Press, M.F., C. Cordon-Cardo & D.J. Slamon: Expression of the HER-2/*neu* proto-oncogene in normal human adult and fetal tissues. *Oncogene* 5, 953-962 (1990)

192. Schroeder, J.A. & D.C. Lee: Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. *Cell Growth Differ* 9, 451-464 (1998)

193. Liu, D., G. Gagliardi, M.R. Alison, T. Oates, E.N. Lalani, G.W. Stamp & M. Pignatelli: TGF-alpha can act as morphogen and/or mitogen in a colon-cancer cell line. *Int J Cancer* 56, 603-608 (1994)



PubMed Nucleotide Protein Genome Structure PMC Taxonomy OMIM Bi

Search PubMed for Go Clear

Limits Preview/Index History Clipboard Details

About Entrez

Display Abstract Show: 20 Sort Send to Text

Text Version

Entrez PubMed

Overview
Help | FAQ
Tutorial
New/Noteworthy
E-Utilities

PubMed Services

Journals Database
MeSH Database
Single Citation Matcher
Batch Citation Matcher
Clinical Queries
LinkOut
Cubby

Related Resources

Order Documents
NLM Gateway
TOXNET
Consumer Health
Clinical Alerts
ClinicalTrials.gov
PubMed Central

Privacy Policy

1: Eur J Dermatol. 2000 Oct-Nov;10(7):505-10.

Related Articles, Li

The EGF receptor - an essential regulator of multiple epidermal functions.

Jost M, Kari C, Rodeck U.

Department of Dermatology and Cutaneous Biology, Institute for Molecular Medicine and the Kimmel Cancer Center, Thomas Jefferson University, 319 BLSB233 S. 10th St., Philadelphia, PA 19107, USA.

Epidermal keratinocytes express both the epidermal growth factor receptor (EGFR) and several of its ligands, establishing the constitutive elements of an autocrine loop in this cell type. Activation of the EGFR provides signals essential to several aspects of normal keratinocyte biology including cell cycle progression, differentiation, cell movement and cellular survival. It may be argued that enhanced keratinocyte survival via EGFR activation is the most important function as it limits the manifestation of other phenotypes. The frequent deregulation of EGFR expression and activation in benign and malignant hyperproliferative skin diseases motivates the investigation of EGFR-dependent intracellular pathways which contribute to the varied EGFR dependent phenotypes.

Publication Types:

- Review
- Review, Tutorial

PMID: 11056418 [PubMed - indexed for MEDLINE]

Display Abstract Show: 20 Sort Send to Text

[Write to the Help Desk](#)

[NCBI](#) | [NLM](#) | [NIH](#)

[Department of Health & Human Services](#)
[Freedom of Information Act](#) | [Disclaimer](#)

Jul 8 2003 10:5

CASE REPORT

Follicular and epidermal alterations in patients treated with ZD1839 (Iressa), an inhibitor of the epidermal growth factor receptor

R. VAN DOORN, G. KIRTSCHIG, E. SCHEFFER,* T. J. STOOF AND G. GIACCONE†

Departments of Dermatology, *Pathology and †Medical Oncology, Vrije Universiteit Medical Centre, PO Box 7057, 1007 MB Amsterdam, the Netherlands

Accepted for publication 18 February 2002

Summary

We report the cutaneous side-effects of ZD1839 (Iressa), a new anticancer agent that acts by inhibiting epidermal growth factor (EGF) receptor signal transduction. Three patients receiving ZD1839 developed an eruption consisting of follicular papules and pustules in an acneiform distribution as well as diffuse fine scaling of the skin. Additionally, hair growth abnormalities were noted in two patients. Histologically, a superficial purulent folliculitis and disordered differentiation with focal parakeratosis were seen. The follicular eruption appeared to respond favourably to treatment with tretinoin cream and minocycline. The cutaneous adverse effects of ZD1839 are similar to those of other EGF receptor-targeted agents and result from direct interference with the functions of EGF receptor signalling in the skin.

Key words: acneiform eruption, cutaneous adverse effects, epidermal growth factor receptor, ZD1839 (Iressa)

ZD1839 (Iressa) is an orally active, selective epidermal growth factor (EGF) receptor tyrosine kinase inhibitor that blocks signal transduction pathways implicated in the proliferation and survival of cancer cells and other host-dependent processes promoting cancer growth.^{1–4} EGF receptors are expressed on various cancer cells, as well as on epidermal keratinocytes and other cells residing in the skin. EGF receptor signalling contributes to cancer growth primarily by inducing proliferation, but also by promoting cell migration and angiogenesis.⁵ ZD1839 selectively inhibits EGF receptor signal transduction by interfering with binding of adenosine triphosphate to the intracellular tyrosine kinase region of this receptor. It is currently being tested in phase II/III clinical trials in the treatment of a number of solid tumours, in particular non-small cell lung cancer. A number of patients included in these trials and treated with oral ZD1839 250 or 500 mg daily developed a similar cutaneous reaction pattern.

Case reports

We examined three patients who experienced cutaneous adverse effects during treatment with ZD1839. In all three patients the cutaneous reaction started 5–12 days after initiation of treatment. Patients developed numerous discrete small pustules and erythematous papules confined to the hair follicles distributed over the face, trunk and upper arms. This was followed by diffuse fine scaling of the interfollicular epidermis involving the whole integument, giving a xerosis-like appearance. No patient had nodular or cystic lesions, and notably there was no seborrhoea. The lesions healed without formation of scars. Patients complained of dry skin and mild to severe itching. None used other medications known to trigger or exacerbate acneiform eruptions. In the three patients described, the cutaneous adverse effects did not necessitate reduction of the dose or discontinuation of treatment with ZD1839.

In patient 1, a 59-year-old man treated with either 250 or 500 mg daily (blinded) ZD1839 as

Correspondence: Dr R. van Doorn.
E-mail: remcovandoorn@hotmail.com

monotherapy, the acneiform eruption began on the face and spread peripherally within weeks to involve the abdomen, forearms and lower legs. Scaling was severe and asteatotic eczema was seen on the legs and flanks. His scalp hair had changed and was curled, fine and brittle. After temporary discontinuation of treatment for reasons other than cutaneous toxicity, the lesions healed without scarring, but reappeared soon after treatment was recommenced. The follicular lesions responded favourably to treatment with emollients and minocycline 100 mg daily.

In patient 2, a 54-year-old woman treated with either 250 or 500 mg daily (blinded) ZD1839 as monotherapy, in addition to follicular papules and pustules, open comedones were present on the chest and back (Fig. 1). After progressive worsening of the eruption during the first 2 weeks of treatment, a spontaneous improvement was noted. As observed in the first patient, the hair was finer, more brittle and curlier, especially on the extremities. She also complained of fragility and easy bruising of the skin. We treated her with tretinoin 0.025% cream, which reduced the development of pustules and comedones, and allowed continued administration of ZD1839.

Patient 3, a 59-year-old man, received ZD1839 500 mg daily in combination with a standard regimen of gemcitabine and cisplatin. The follicular lesions became confluent, forming erythematous macules and plaques studded with pustules on the forehead (Fig. 2). On the upper back both comedones and follicular papules were seen. This patient noticed that the growth of his beard had slowed down during the treatment. Twice daily application of tretinoin 0.025% cream led

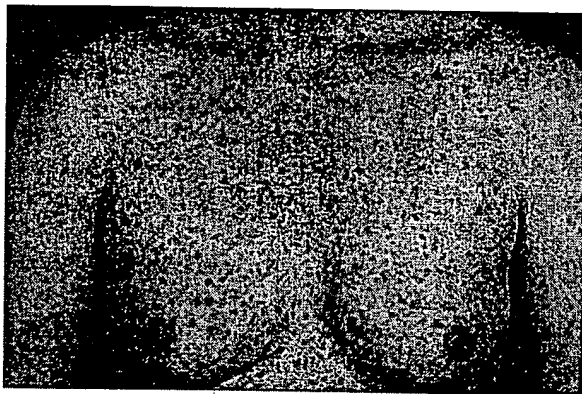


Figure 1. Follicular eruption on the chest of a 54-year-old woman treated with ZD1839 (patient 2).

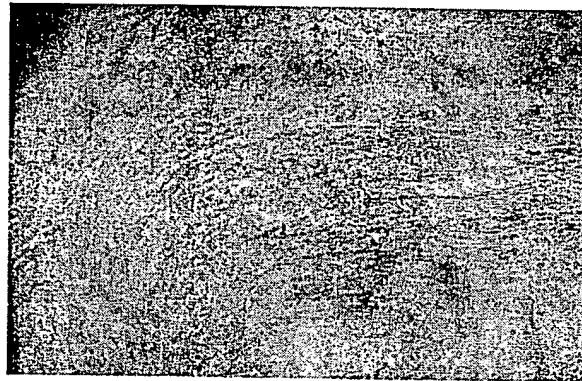


Figure 2. Pustules on an erythematous base as well as scaling of the interfollicular epidermis on the forehead of a 59-year-old man treated with ZD1839 500 mg daily in combination with a standard regimen of gemcitabine and cisplatin (patient 3).

to improvement of the follicular lesions, but aggravated the scaling of the skin.

Laboratory investigations

Bacterial and fungal cultures of the pustular contents showed *Propionibacterium acnes* in patient 2 and absence of pathogenic micro-organisms in patients 1 and 3. The total blood count did not show eosinophilia or other alterations in any patient.

Histology

Three skin biopsies taken from pustular lesions of patients 1 and 3 showed similar histological changes. Different stages of a purulent folliculitis were seen. Most follicles were surrounded by an infiltrate composed of lymphocytes and histiocytes. The superficial portion of some follicles was densely infiltrated by neutrophilic granulocytes with partly fragmented nuclei and histiocytes (Fig. 3). The formation of follicular pustules was often accompanied by a remarkable absence of the follicular epithelial lining. In more advanced lesions there was destruction of the follicle with perifollicular granuloma formation, dermal oedema and vasodilatation. The sebaceous glands were relatively small and were not affected by the infiltrate. No micro-organisms were found. The epidermis showed more subtle changes: the stratum corneum had lost its basket-weave appearance, and was thin and compact with varying parakeratosis. There was somewhat irregular epidermal differentiation with slight hypogranulosis, but no evident atrophy.

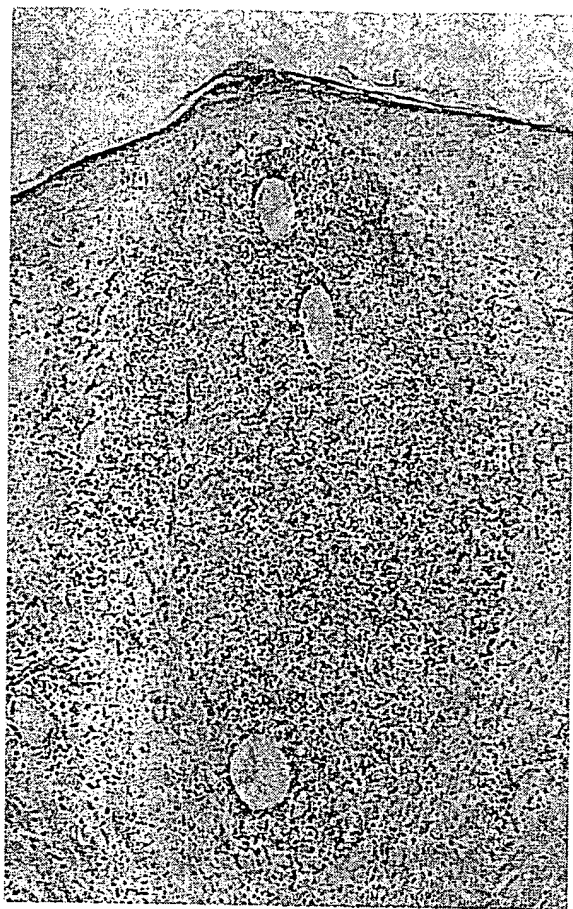


Figure 3. Photomicrograph from a pustule on the back, demonstrating a follicle filled with partly degenerated neutrophilic granulocytes and histiocytes (patient 1; haematoxylin and eosin; original magnification $\times 10$).

Discussion

The skin eruption observed in these patients treated with the EGF receptor inhibitor ZD1839 consisted of follicular papules and pustules in an acneiform distribution and diffuse fine scaling of the skin. Hair growth abnormalities were noted in two patients. Histology of the skin lesions revealed a purulent folliculitis as well as slightly irregular epidermal differentiation. Similar cutaneous adverse effects have been observed in other patients treated with EGF receptor-targeted therapies. In a phase II trial of the EGF receptor tyrosine kinase inhibitor OSI-774, a maculopapular acneiform rash was observed in 78% of patients.⁶ In addition, cancer patients treated with C225 (cetuximab), an anti-EGF receptor monoclonal antibody, developed an acneiform follicular eruption very similar to that seen in patients

treated with ZD1839.^{7,8} In patients treated with ZD1839 it has been reported that the frequency of development of the pustular eruption is dose-dependent.⁹ These observations support our conclusion that the cutaneous adverse effects seen in these patients are not primarily immunologically mediated but represent the results of inhibition of EGF receptor signal transduction in epidermal and follicular epithelium. This cutaneous reaction pattern therefore reflects the significance of the EGF signalling pathway in skin.

In human skin the EGF receptor is expressed by basal epidermal keratinocytes, outer root sheath cells and sebocytes.¹⁰ Its activation by several keratinocyte-derived ligands stimulates proliferation and reduces the susceptibility to apoptosis.¹¹ Furthermore, in the epidermis activation of the EGF receptor reduces the terminal differentiation capacity of basal keratinocytes, but promotes differentiation of suprabasal keratinocytes.¹² Inhibition of the EGF receptor tyrosine kinase *in vitro* induces human keratinocyte growth arrest and terminal differentiation.¹³ The epidermal alterations demonstrated in our patients, clinically apparent as diffuse fine scaling, are consistent with these *in vitro* findings in that they reflect a disturbance of the equilibrium between proliferation and differentiation.

In the hair follicle the EGF receptor-ligand system has an essential role in regulation of the hair cycle, as activation of the EGF receptor stimulates transition from anagen to catagen.¹⁴ Mice harbouring a targeted disruption of the EGF receptor allele display short and wavy hair that becomes progressively atrophic, eventually resulting in alopecia.^{15,16} The hair follicles in these mice do not progress from anagen to telogen and are specifically destroyed by an inflammatory infiltrate. The hair growth abnormalities and folliculitis observed in some patients treated with ZD1839 display an analogy to the findings in these transgenic mice and could thus result from inhibition of hair cycle progression. However, the precise mechanism by which inhibition of EGF receptor signalling induces a purulent folliculitis has yet to be elucidated.

The follicular eruption induced by ZD1839 has many similarities with acne vulgaris and we therefore decided to treat it empirically with tretinoin cream in two patients and minocyclin in one patient. The follicular eruption responded favourably to both of these treatments, although application of tretinoin cream worsened the scaling of the skin.

Further studies are warranted to elucidate the mechanisms by which EGF receptor-targeted therapies, such as ZD1839, induce this cutaneous reaction,

firstly to permit a more natural approach to its treatment and secondly because it could provide information on the physiological role of EGF receptor signalling in human skin. In addition, the possible use of ZD1839 in the treatment of skin diseases in which activity of the EGF receptor-ligand system is increased, such as certain skin tumours and psoriasis, deserves further attention.

Acknowledgments

We thank Prof Dr T.M.Starink for reviewing the histological sections. We thank N.Kok, R.Ruiter, I.Zegers and S.D.Jones (AstraZeneca Netherlands BV) for their co-operation.

References

- Baselga J, Averbuch SD. ZD1839 ('Iressa') as an anticancer agent. *Drugs* 2000; **60** (Suppl. 1): 33–40.
- Meric JB, Faivre S, Monnerat C *et al.* ZD 1839 'Iressa'. *Bull Cancer* 2000; **87**: 873–6.
- Baselga J. New therapeutic agents targeting the epidermal growth factor receptor. *J Clin Oncol* 2000; **18** (Suppl. 21): 54–9.
- Raymond E, Faivre S, Armand JP. Epidermal growth factor receptor tyrosine kinase as a target for anticancer therapy. *Drugs* 2000; **60** (Suppl. 1): 15–23.
- Ciardiello F, Caputo R, Bianco R *et al.* Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin Cancer Res* 2001; **7**: 1459–65.
- Perez-Soler R, Chachoua A, Huberman M *et al.* A phase II trial of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor OSI-774, following platinum-based chemotherapy, in patients (pts) with advanced, EGFR-expressing, non-small cell lung cancer (NSCLC). *Proceedings of the ASCO* 2001; **20** (Abstr. 1235).
- Busam KJ, Capodiceci P, Motzer R *et al.* Cutaneous side-effects in cancer patients treated with the antiepidermal growth factor receptor antibody C225. *Br J Dermatol* 2001; **144**: 1169–76.
- Kimyai-Asadi A, Jih MH. Follicular toxic effects of chimeric anti-epidermal growth factor receptor antibody cetuximab used to treat human solid tumors. *Arch Dermatol* 2002; **138**: 129–31.
- Kris M, Ranson M, Ferry D *et al.* Phase I study of oral ZD1839 (Iressa™), a novel inhibitor of epidermal growth factor receptor tyrosine kinase (EGF-TK): evidence of good tolerability and activity. *Proceedings of the AACR NCI EORTC*, 1999; **21**: (Abstr. 99).
- Nanney LB, Magid M, Stoscheck CM, King LE Jr. Comparison of epidermal growth factor binding and receptor distribution in normal human epidermis and epidermal appendages. *J Invest Dermatol* 1984; **83**: 385–93.
- Jost M, Kari C, Rodeck U. The EGF receptor, an essential regulator of multiple epidermal functions. *Eur J Dermatol* 2000; **10**: 505–10.
- Wakita H, Takigawa M. Activation of epidermal growth factor receptor promotes late terminal differentiation of cell–matrix interaction-disrupted keratinocytes. *J Biol Chem* 1999; **274**: 37285–91.
- Peus D, Hamacher L, Pittelkow MR. EGF-receptor tyrosine kinase inhibition induces keratinocyte growth arrest and terminal differentiation. *J Invest Dermatol* 1997; **109**: 751–6.
- Philpott MP, Sanders D, Westgate GE, Kealey T. Effects of EGF on the morphology and patterns of DNA synthesis in isolated human hair follicles. *J Invest Dermatol* 1994; **102**: 186–91.
- Murillas R, Larcher F, Conti CJ *et al.* Expression of a dominant negative mutant of epidermal growth factor receptor in the epidermis of transgenic mice elicits striking alterations in hair follicle development and skin structure. *EMBO J* 1995; **14**: 5216–23.
- Hansen LA, Alexander N, Hogan ME *et al.* Genetically null mice reveal a central role for epidermal growth factor receptor in the differentiation of the hair follicle and normal hair development. *Am J Pathol* 1997; **150**: 1959–75.

Agensys

ORIGINAL

1545 17th Street
SANTA MONICA, CA 90404
(310) 820-8029 OFFICE
(310) 820-7880 FAX

FAX COVER SHEET

TO: David Devernoe, Esq.
FAX #: 858.720.5125
FROM: Timothy Lithgow, M.D., J.D., Senior Director Intellectual Property
DATE: Thursday, July 10, 2003
RE: 51158-20035.00

Pages including cover: 7

MESSAGE: **Faris Dec. Executed**

THIS MESSAGE IS INTENDED ONLY FOR THE USE OF THE INDIVIDUAL OR ENTITY TO WHICH IT IS ADDRESSED, AND MAY CONTAIN INFORMATION THAT IS PRIVILEGED, CONFIDENTIAL AND EXEMPT FROM DISCLOSURE UNDER APPLICABLE LAW. IF THE READER OF THIS MESSAGE IS NOT THE INTENDED RECIPIENT, OR THE EMPLOYEE OR AGENT RESPONSIBLE FOR DELIVERING THE MESSAGE TO THE INTENDED RECIPIENT, YOU ARE HEREBY NOTIFIED THAT ANY DISSEMINATION, DISTRIBUTION OR COPYING OF THIS COMMUNICATION IS STRICTLY PROHIBITED. IF YOU HAVE RECEIVED THIS COMMUNICATION IN ERROR, PLEASE NOTIFY US IMMEDIATELY BY TELEPHONE AND RETURN THE ORIGINAL MESSAGE TO US AT THE ABOVE ADDRESS VIA THE U.S. POSTAL SERVICE. THANK YOU.

PATENT
Docket No. 511582003500

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Mary FARIS, et al.

Serial No.: 09/809,638

Filing Date: March 14, 2001

For: 125P5C8: A TISSUE SPECIFIC
PROTEIN HIGHLY EXPRESSED IN
VARIOUS CANCERS

Examiner: Alana M. Harris, Ph.D.

Group Art Unit: 1642

**DECLARATION OF MARY FARIS, PH.D.
CONCERNING NORMAL TISSUE EXPRESSION**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Mary Faris, declare as follows:

1. I hold the position of Group Leader-Target Validation at Agensys, Inc. This position requires me to generate *in vitro* models for the study of cancer, and to investigate the effect of specific genes and gene products on tumor development, growth, and progression. This position also requires me to attend national and international conferences addressing issues in cancer research, conferences where established as well as cutting edge ideas are presented. I have a Ph.D. in Immunology and Microbiology from Ohio State University. In addition, I have held two postdoctoral fellowships, one at the University of Virginia and one at the University of California at Los Angeles, School of Medicine. I have worked in the field of molecular biology

academically and professionally for over 15 years. A copy of my *curriculum vitae* is attached as Exhibit A.

2. This submission is made in support of the proposition that targeted antitumor therapies are useful even when the targeted protein is expressed on normal tissues, what is more even if the normal tissue is from a vital organ. A vital organ is one that is necessary to sustain life, such as the heart or brain. A non-vital organ is one that can be removed whereupon the individual is able to survive without artificial life support. Examples of non-vital organs are ovary, breast, and prostate. The expression and targeting of two exemplary proteins will be discussed, HER2/neu and epidermal growth factor receptor (EGFR).

3. Herceptin® is an FDA approved pharmaceutical that has as its active ingredient an antibody which is immunoreactive with the protein known synonymously as HER2, HER2/neu, or erb-b-2. It is marketed by Genentech and has been a commercially successful antitumor agent. Herceptin sales reached almost \$400 million in 2002 (see Exhibit B). The success of Herceptin is indicated by both the volume and trend of its sales figures. During the period it has been marketed, its sales numbers have consistently increased.

4. Herceptin is FDA approved as a treatment for HER2 positive metastatic breast cancer (see Exhibit C). However, the expression of HER2 is not limited to such tumors. The same protein is expressed in a number of normal tissues.

5. The diverse expression of the HER2/neu protein is indicated in Exhibit D.¹ Exhibit D provides data panels for three different GenBank deposits of HER2/neu sequence. The comparative data in Exhibit D show levels of HER2 above the median in numerous normal tissues. In particular, attention is called to the levels present in kidney and heart. The levels in

kidney are consistently shown as higher than those in heart. It is emphasized that these values are for normal tissue; thus these tissues are present in *all human recipients* of Herceptin.

6. The expression of HER2/neu in a diverse array of normal and malignant tissues was corroborated by studies conducted at Agensys, assignee of the present case, by use of RT-PCR (Exhibit F). Again, significant expression of HER2/neu was noted in heart and kidney. Further corroboration of normal expression of HER2/neu by immunohistochemistry is set forth in Exhibit G. In Exhibit G, positive staining for the presence of HER2/neu was found in normal kidney and colon tissue. Additional confirmation of the presence of HER2/neu in normal kidney is provided by Latif, et al.² As shown in this article (which evaluated whether renal cell carcinoma should be a preferred indication for anti-HER2 antibodies such as Herceptin), both protein and mRNA are produced in benign renal tissues. Notably, HER2/neu protein was strongly overexpressed in benign renal tissue (see page 8, left hand column last paragraph of Latif, et al.).

7. Despite the fact that HER2/neu is expressed in vital organs such as heart and kidney, Herceptin is very useful, FDA-approved, and commercially successful. The effect of Herceptin on cardiac tissue, i.e., "cardiotoxicity," has been a limited side effect to treatment. When patients were treated with Herceptin alone, a very low percentage of patients had any significant cardiotoxicity.

8. Of particular note, although the data shows that kidney tissue exhibits even higher expression than cardiac tissue, nephrotoxicity has not been an appreciable side effect of Herceptin treatment at all. In fact, of the diverse normal tissues in which HER2 is expressed,

¹ The expression data in Exhibit D was established using gene chip technology; see Su, et al, *PNAS Proc. Natl. Acad. Sci. USA*, Vol. 99, Issue 7, 4465-4470, April 2, 2002, Exhibit E. The results for HER2 are available on the GNF Gene Expression Atlas website <http://expression.gnf.org/cgi-bin/index.cgi>.

² Latif, Z., et al., *B.J.U. International* (2002) 89:5-9 (Exhibit H)

there is very little occurrence of any side effect. Only cardiac tissue has any appreciable side effect at all. A tissue such as kidney, where HER2/neu expression is appreciable, has not been the locus for any side effect.³

9. Taken together, this documentation establishes that production of a target protein such as HER2/neu on normal tissue, even vital normal tissue, does not defeat the utility of the protein as a therapeutic for certain tumors in which the protein is also expressed.⁴ Such physiologic outcomes, where there is normal tissue expression of a cancer-associated protein target, are not unique to HER2/neu as will be discussed below.

10. Several anti-cancer therapeutic products that target the epidermal growth factor receptor (EGFR) are presently in clinical evaluation.⁵ The small molecule product Iressa® received FDA approval⁶ in May 2003. The rationale for EGFR-targeted anti-cancer treatments is both well known and well accepted.⁷

11. One EGFR-targeted anti-cancer treatment composition is Erbitux™ (also known as cetuximab or C225). The active ingredient in Erbitux is an antibody which is immunoreactive with the EGFR. Erbitux antibody has been shown to block the proliferation of various cancer cells.⁸ The successful use of Erbitux is shown, for example, by a March 2003 \$60 million commercialization payment (Exhibit K), as well as very exciting European clinical trial data disclosed in early 2003.⁹ This data indicated that Erbitux was able to *affect shrinkage of tumor*

³ See Exhibit C, the product information for Herceptin® for an overview of side effect issues

⁴ This is particularly true where the target protein, as with HER2/neu is expressed at higher levels in tumor cells relative to normal tissue. In one embodiment, overexpression in tumor tissue can provide for altered, e.g., enhanced, protein availability for antibody binding.

⁵ Baselega, J., *Oncologist* 7 (supp. 4):2-8 (2002) (Exhibit I)

⁶ Iressa attaches itself to the EGF receptor inside the cell, which blocks the activation of tyrosine kinase, and switches off the signals from the EGFR.

⁷ Id.

⁸ Busam, et al. *Br. J. Derm.* 144:1169-1176 at 1169 (2001) (Exhibit J)

⁹ C. Arnst "The War on Cancer: Good News, Glum Faces" BusinessWeek Online, June 3, 2003 <http://www.businessweek.com/technology/content/jun2003/tc2003063_1032.htm> (Exhibit K)

size by 50%. Tumor size was decreased by 50% in 11% of patients who received Erbitux as a single agent and in 23% of patients who received Erbitux in combination with chemotherapy.¹⁰ In addition, tumor progression was delayed and life span appeared to be increased in the treated cancer patients. These are significant findings for a patient population with dire prognosis, few if any treatment options, and very short life expectancy.

12. EGFR is therefore being used and evaluated as a target for treatment of patients with breast, head and neck, lung, kidney and prostate cancer.¹¹ However, the expression of EGFR is not limited to such tumors. This protein is expressed in a diverse array of normal tissues.

13. EGFR protein is extensively expressed in adult humans. It is present on all epithelial and stromal cells, select glial and smooth muscle cells,¹² oral and laryngeal mucosa¹³; brain;¹⁴ liver,¹⁵ prostate;¹⁶ placenta;¹⁷ stomach and colon;¹⁸ and, skin.¹⁹ It is to be noted that since it is expressed in these normal tissues, EGFR is present in all human recipients of any EGFR-targeted therapy.

14. Despite the fact that EGFR is expressed in numerous normal tissues, including vital tissues such brain and colon, therapeutics that target EGFR are very useful and are in active development. The only significant side effect of such therapeutics has been on skin as an

¹⁰ "Study backs ImClone drug's effectiveness" American Society of Clinical Oncology (ASCO) web page, June 2, 2003 <http://www.asco.org/ac/1,1003,12-002122-00_18-0028202-00_19-0028203-00_20-001,00.asp> (Exhibit L)

¹¹ Busam, et al, *supra*

¹² Wells, A., *Int. Biochem & Cell Biol.* 31:637-643 at 640 (1999) (Exhibit M)

¹³ Christensen, M., *Dan Med Bull.* 45(2):121-134 (Apr. 1998) (Exhibit N)

¹⁴ Ferrer, et al., *Prog. Neurobiol.* 49(2):99-123 (Jun. 1996) (Exhibit O)

¹⁵ Luwar, et al., *Cancer Res.* 61:5355-5361 (July 15, 2001) (Exhibit P)

¹⁶ De Miguel, et al., *Cytokine* 11(9):722-727 (Sep 1999) (Exhibit Q)

¹⁷ Mauro, et al., *Repro. Fertil. Devel.* 7(6): 1465-1470 (1995) (Exhibit R)

¹⁸ Challier and Menard, *Frontiers in Biosci.* 4:87-101 at sections 4.2 and 4.3 (January 15, 1999) (Exhibit S)

¹⁹ Jost, et al. *Eur. J. Dermatol.* 10(7):505-510 (Oct-Nov 2000); Luwar, et al., *Cancer Res.* 61:5355-5361 (July 15, 2001) (Exhibit T)

acneiform rash.²⁰ This has merely been a side effect to treatment. This side effect is so minor that it is used as an indication that therapeutically effective dosage levels have been achieved.²¹ Notably, the side effect is actually capitalized on as a relatively innocuous signal that an individual has achieved a therapeutically effective dose.

15. The data on EGFR establishes that production of a target protein such as EGFR on normal tissue, even vital normal tissues, does not preclude the utility of the protein as a therapeutic for certain tumors in which the protein is also highly expressed.²²

16. Thus, targeted antitumor therapies are useful even when the targeted protein is expressed on normal tissues, even normal vital organ tissues. The ability to use a cancer-associated protein in this manner is not unique to any particular protein. The existence of expression of a protein on a normal tissue, even a vital organ tissue, still allows for meaningful and successful use of that protein as a therapeutic target.

17. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are

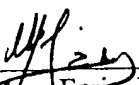
²⁰ Busam, et al, *supra*; Van Doorn, et al., Br. J. Derm. 147:598-601 (2002) (Exhibit U)

²¹ Abgenix Press release 20 Aug 2002 <<http://ir.abgenix.com/phoenix.zhtml?c=91622&p=IROL-NewsText&t=Regular&id=381536>> (Exhibit V)

²² See footnote 4, *supra*

punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at Santa Monica, California, this 9th day of July 2003.



Mary Faris, Ph.D.

Abgenix Reaffirms Optimism Regarding ABX-EGF

FREMONT, Calif.--(BUSINESS WIRE)--Aug. 20, 2002--Abgenix, Inc. (Nasdaq:ABGX - News) commented today on the unusually large volume of trading in its common stock yesterday, apparently triggered by Astra Zeneca's announcement that its small-molecule drug, Iressa, does not provide improvement in survival when added to standard chemotherapy versus chemotherapy alone in the first line treatment of advanced non-small cell lung cancer (NSCLC). Abgenix' fully human monoclonal antibody product candidate, ABX-EGF, is currently being tested in clinical trials both as monotherapy and in combination with standard chemotherapy in several types of cancer, including colorectal cancer and NSCLC. Due to the following differences between ABX-EGF and the small molecule drugs targeting the epidermal growth factor receptor (EGFr), the company believes that expectations about the results of the ongoing ABX-EGF trials should not be based on the Iressa results announced yesterday. ABX-EGF has not shown dose limiting toxicity, while the small molecule drugs targeting the EGFr are dose-limited by the occurrence of severe diarrhea. The current dose of ABX-EGF has been set at the level that results in 100% of patients achieving an acneiform skin rash that suggests full blockade of the EGFr. ABX-EGF has demonstrated low pharmacokinetic inter-patient variability resulting in consistent exposure of each patient to the drug. Small molecule drugs targeting the EGFr pathway are eliminated from the body by enzymes, such as p450, that are also involved in the elimination of some small molecule chemotherapy drugs. Therefore other small molecule drugs may interfere with the rate at which a small molecule EGFr-targeting agent is eliminated, but are highly unlikely to interfere with the rate of elimination of an antibody, which leaves the body by a different mechanism (mediated by the EGF receptors). In early phase studies ABX-EGF has shown single agent biological activity in patients with advanced renal cell cancer. "We continue to believe in the importance of the EGF receptor as a target for drug development," said Raymond Withy, Ph.D., president and chief executive officer of Abgenix. "We remain optimistic about the potential of ABX-EGF for patients with a variety of cancer types and look forward to the upcoming results of our ongoing clinical trials." ABX-EGF is a fully human monoclonal antibody generated using XenoMouse(TM) technology that targets the EGFr, which is over-expressed in a variety of cancers including lung, breast, bladder, prostate, colorectal, kidney and head and neck cancer. It has been demonstrated that cancer cells can become dependent on growth signals mediated through the EGFr for their survival. In preclinical research, ABX-EGF monotherapy has been shown to both eradicate established human tumors and block the growth of human tumors. ABX-EGF is being co-developed by Abgenix, Inc. and Amgen Inc. and is currently being evaluated in a comprehensive Phase 2 program in several indications including kidney, non-small cell lung, colorectal and prostate cancer.

Abgenix is a biopharmaceutical company focused on the development and commercialization of human therapeutic antibodies. The company's technology platform, which includes XenoMouse® and XenoMax(TM) technologies, enables the rapid generation and selection of high affinity, fully human antibody product candidates to a variety of disease targets. Abgenix leverages its leadership position in human antibody technology by building a diversified product portfolio through the development of its own internal proprietary products and through the establishment of licensing arrangements with multiple pharmaceutical, biotechnology and genomics companies. For more information on Abgenix, visit the company's website at www.abgenix.com. Statements made in this press release about Abgenix's technologies, product development activities and collaborative arrangements other than statements of historical fact, are forward looking statements and are subject to a number of uncertainties that could cause actual results to differ materially from the statements made, including risks associated with the success of clinical trials, the progress of research and product development programs, the regulatory approval process, competitive products, future capital requirements and the extent and breadth of Abgenix's patent portfolio. Please see Abgenix's public filings with the Securities and Exchange Commission for information about risks that may affect Abgenix. Contact: Abgenix, Inc. Ami Knoeffler, 510/284-6350

###

TOP ^

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.